Available online at www.sciencedirect.com
SCIENCE DIRECT
ELSEVIER
Journal of Chromatography A, 1099 (2005) 1–42
JOURNAL OF CHROMATOGRAPHY A
www.elsevier.com/locate/chroma

Review

Critical contribution of nonlinear chromatography to the understanding of retention mechanism in reversed-phase liquid chromatography

Fabrice Gritti [a,b], Georges Guiochon [a,b,*]

[a] *Department of Chemistry, University of Tennessee, Knoxville, TN 37996-1600, USA*
[b] *Division of Chemical Sciences, Oak Ridge National Laboratory, Oak Ridge, TN 37831-6120, USA*

Received 21 March 2005; received in revised form 23 September 2005; accepted 27 September 2005
Available online 2 November 2005

Abstract

The retention of most compounds in RPLC proceeds through a combination of several independent mechanisms. We review a series of recent studies made on the behavior of several commercial C_{18}-bonded stationary phases and of the complex, mixed retention mechanisms that were observed in RPLC. These studies are essentially based on the acquisition of adsorption isotherm data, on the modeling, and on the interpretation of these data. Because linear chromatography deals only with the initial slope of the global, overall, or apparent isotherm, it is unable fully to describe the complete adsorption mechanism. It cannot even afford clues as to the existence of several overlaid retention mechanisms. More specifically, it cannot account for the consequences of the surface heterogeneity of the packing material. The acquisition of equilibrium data in a wide concentration range is required for this purpose. Frontal analysis (FA) of selected probes gives data that can be modeled into equilibrium isotherms of these probes and that can also be used to calculate their adsorption or affinity energy distribution (AED). The combination of these data, the detailed study of the best constants of the isotherm model, the determination of the influence of experimental parameters (e.g., buffer pH and p*I*, temperature) on the isotherm constants provide important clues regarding the heterogeneity of the adsorbent surface and the main properties of the adsorption mechanisms. The comparison of similar data obtained for the adsorption of neutral and ionizable compounds, treated with the same approach, and the investigation of the influence on the thermodynamics of phase equilibrium of the experimental conditions (temperature, average pressure, mobile phase composition, nature of the organic modifier, and, for ionizable compounds, of the ionic strength, the nature, the concentration of the buffer, and its pH) brings further information. This review provides original conclusions regarding retention mechanisms in RPLC.

Keywords: RP-HPLC; Retention mechanism; Adsorption isotherm; Frontal analysis; Adsorption energy distribution; Adsorption data reproducibility; Column heterogeneity; Organic modifier; Temperature; Pressure; Ionizable compounds

Contents

* Corresponding author. Tel.: +1 865 974 0733; Fax: +1 865 974 2667.
E-mail address: guiochon@utk.edu (G. Guiochon).

0021-9673/$ – see front matter © 2005 Elsevier B.V. All rights reserved.
doi:10.1016/j.chroma.2005.09.082

1. Introduction

The surface of all modified adsorbents is heterogeneous. This phenomenon arises from the tendency of the elemental impurities of the material making the bulk of the adsorbent (e.g., alumina, boron or iron in silica) to segregate at the surface. Foreign elements are more concentrated on the surface of adsorbents than in their bulk and only high purity adsorbents have relatively homogeneous surfaces. Furthermore, the presence of the surface itself generates stresses and strains of the bonds involving polyvalent atoms located at the solid interface or in its immediate vicinity. The presence of various element impurities on the surface and these bond strains cause a heterogeneous distribution of the adsorption energy (AED) of different probes. The properties of this distribution depend on the nature of the probe used and the adsorbent considered. On the adsorbent the surface of which is closest to an ideally homogeneous one, graphitized carbon black, there are occasional steps between successive 0 0 1 planes. In contrast, the surfaces of the porous silica particles used as the base material for the preparation by chemical bonding of suitable ligands of the packing materials used in reversed-phase (RPLC) or hydrophobic interaction (HIC) chromatography, are highly heterogeneous. The profile of the AED of a probe, particularly the number, the width, and the position of its modes, may characterize the degree of heterogeneity of the surface. The AEDs of different probes on a given surface are different since the interactions of these probes with the surface depend largely on the nature of the probe. The nature of the AED explains the complexity of the equilibrium isotherm of a probe between a solid adsorbent and a solution. This phenomenon is generally known in chromatography as mixed retention mechanisms. Its consequences are particularly insidious in RPLC.

RPLC has become the most popular mode of implementation of elution HPLC. It owes this preeminent position to the availability of highly pure microparticles of silica that can be bonded to a variety of non polar or moderately polar groups,[1] giving packing materials of great chemical stability. The availability of these materials a score ago was a considerable progress over those of the early ages of liquid–liquid chromatography [1–4]. Twenty-five years ago, already 80–90% of HPLC analyses were carried out with columns packed with these chemically bonded silica materials [5]. It seems that this proportion has remained the same.

The products of the most important chemical, biomedical, food, and pharmaceutical industries are produced, sold and delivered on the basis of RPLC analytical results. This wide range of applications of RPLC, in turn, has contributed to the development of an important group of companies making modern RPLC columns. Manufacturers keep preparing and developing

[1] If the group is polar, the separation is not made in RPLC and this topic is outside of the scope of this review.

new stationary phases with higher mechanical strength, better reproducibility, faster mass transfer kinetics and higher efficiency, broader pH range stability, higher selectivity (whatever way it is defined), etc. The last decade has seen the rapid emergence of new solid supports, e.g., polymer particles, hybrid particles, silica particles covered by a complex grafted organic layer. Although these products seem in general to perform better and better, our understanding of the retention mechanism(s) involved in a separation has not much improved.

The retention of analytes in RPLC is fundamentally determined by their distribution between a liquid polar mobile phase and an apolar stationary phase consisting of an organic layer, most often made of alkyl chains but sometimes of a hydrocarbon polymer, that is bonded to the silica surface. This equilibrium is not strictly a liquid–solid equilibrium because the solid adsorbent is covered by a chemically modified hydrophobic layer which has a finite thickness, up to 30 Å for C_{30}-bonded phases. Because of the relative mobility of these chains, this layer cannot be considered as the sharp interface existing at conventional solid–liquid interface. However, it cannot be considered as a liquid either because the alkyl chains are attached to the surface at one of their extremities. In contact with the liquid mobile phase, the layer of bonded alkyl chains adsorbs selectively the components of the mobile phase, swells to a degree, and forms a thick solid–liquid interface which has a complex structure. The physical properties of this interface have not yet been clearly established despite numerous investigations motivated by our suspicion that the understanding of the structure of this hydrophobic layer is the key toward a general understanding of retention mechanisms in RPLC.

An early RPLC retention model was based on the assumption that the formation of a suitable cavity in the mobile phase to accommodate the analyte molecule was the key step in the retention mechanism (solvophobic theory [6]). Accordingly, this model assumed that the retention of a compound depended essentially on its size and on the surface tension of the mobile phase. The limit of this model became obvious when experimental data showed that retention was also governed by the density and the length of the alkyl chains bonded to the silica surface [7–9]. The differences observed were unambiguously interpreted as originating from variations of the phase ratio [10–14]. Long ago, it was suggested that the structure of the bonded layer is such that it could explain the simultaneous presence of adsorption and partition sites [15,16]. Thus, new models which include the characteristics of both the mobile and the stationary phases were elaborated. The partitioning model [17,18] in which the analyte is transfered from the mobile phase to the stationary phase accounted far better for the experimental data than earlier models. The retention factors of analytes were successfully correlated with their partition coefficients between water and *n*-octane, shown to be proportional to the molecular size of the solute, and to be directly affected by their solubility in the mobile phase. However, the driving force for solute adsorption that was measured appeared smaller than predicted by the partitioning model. So, an adsorption model was proposed instead [17,18]. These two models of the actual retention mechanism, the adsorption and the partition models, are certainly the two extremities of a broad spectrum of possible retention modes in RPLC. This frame of understanding of the retention mechanisms in RPLC is justified by the heterogeneous nature of the adsorption layer and by the complex organization of the interphase layer. It has been demonstrated that alkyl bonded phases are made of ordered and disordered regions, regions that were observed in FTIR [19,20] and in NMR [21–23]. Accordingly, the overall retention of an analyte in RPLC is more the result of a complicated convolution of many different interactions happening simultaneously.

The lack of a clear understanding of the retention mechanism in RPLC is largely due to the fact that practically all investigations of retention mechanisms made so far rely on the acquisition of chromatographic data measured under linear conditions. Most models used in linear chromatography assume that the fundamental equilibrium constant K that describes the distribution of an analyte at infinite dilution between the stationary and the mobile phase is simply the product of elementary equilibrium constants K_i, each one describing a particular type of molecular interactions [24–27]. These interactions, hydrophobic, steric, donor and acceptor hydrogen-bonding, and ion exchange interactions, are supposed to act independently, so:

$$\ln K = \ln \prod_j K_j = \sum_j \frac{-\Delta G_j^\circ}{RT} = \sum_j d_j (a_{s,j} - a_{m,j}) \qquad (1)$$

Each free energy term ΔG_j° is assumed to be the product of the solute descriptor d_j characterizing the type of interactions j and the term $(a_{s,j} - a_{m,j})$ that characterizes the pair of stationary (s) and mobile (m) phase selected.

Such empirical Linear Free Energy Relationships have been extensively used to characterize RP column selectivities and many sets of retention data were successfully fitted to such a model. Although this model takes into account the fact that a solute may simultaneously interact through different types of intermolecular interactions with the stationary phase $(d_j \times a_{s,j})$ and the liquid phase $(d_j \times a_{m,j})$, it fundamentally neglects the facts that (1) the stationary phase might be heterogeneous, hence that the terms $a_{s,j}$ are not necessarily unique but depend on the position on the surface and (2) there might be several different micro-environments in the liquid phase (as is the case with ACN [113]), each having a different $a_{m,j}$ term. As we explain later, all conventional RPLC adsorbents are heterogeneous. If we call i the type of adsorption sites on the stationary phase and if we assume an homogeneous liquid phase, a consistent way of writing the equilibrium constant K would be:

$$K = \frac{C_S}{C_M} = \frac{\sum_{i=1}^{i=N} N_{S,i}/V_S}{C_M} = \sum_{i=1}^{i=N} f_i K_i(T)$$

$$= \sum_i f_i \left(\prod_j K_{i,j} \right) \qquad (2)$$

where $N_{S,i}$ is the amount of compound in the subphase i, f_i the volume fraction of the stationary phase occupied by the sites i, and $K_i(T)$ is the thermodynamic equilibrium constant for the distribution of the compound between the adsorption sites i and the liquid phase.

Accordingly, the extension of the empirical LFER equations to heterogeneous surface would be:

$$\ln K = \ln \left[\sum_i f_i \exp \left(\sum_j d_j (a_{s,i,j} - a_{m,j}) \right) \right] \quad (3)$$

Even though this more complex retention model takes into account the surface heterogeneity of the solid adsorbent, it cannot give much information on the physical nature of the chromatographic system involved, e.g. on the quantity of sites of type i, $q_{s,i}$, and on the adsorption–desorption constant on these sites b_i (with $K_i = q_{s,i} b_i$) nor on the adsorption energies on the sites of type i. The LFER results given by Eq. (1) are empirically fitted parameters that inform on the contribution of each type of interactions j to the overall solute retention. A deconvolution process would be required to separate the contributions of the density of the different types of adsorption sites $q_{s,i}$ and of the adsorption–desorption constant b_i. This can be achieved only if the experimental conditions allow to perform measurements at concentrations approaching the saturation of the different adsorption sites. High concentration samples should be injected successively to saturate these different types of sites. The values of the amount of analyte adsorbed per unit volume of the stationary phase q^* for each concentration injected C will be related to the saturation capacities $q_{s,i}$ and the thermodynamic equilibrium constants $K_i(d_j, a_{s,i,j}, a_{m,j})$. Assuming the formation of an adsorbed monolayer without lateral interactions between adsorbate molecules on each type of sites, a general multi-Langmuir adsorption isotherm can be written as:

$$q^*(C) = \sum_i q_{s,i} \frac{K_i(d_j, a_{s,i,j}, a_{m,j}) C}{q_{s,i} + K_i(d_j, a_{s,i,j}, a_{m,j}) C} \quad (4)$$

Such a model could be further applied in the same way as the LFER is applied to linear data, in order to characterize completely the chromatographic system (quantity of sites i, contribution of each type of interactions j and overall adsorption energy on each site i). There are significant new experimental constraints linked to this approach, arising from a limited solubility of the analyte, possible adsorbate–adsorbate interactions, and solute–solute association (different forms of the same specie being then present in the system). The resolution of the problem remains the same in principle, but more sophisticated adsorption models could be considered instead.

As aforementionned, the retention factors derived are the sum of the different contributions (or Henry constants) originating from the different sites available on the surface of or in the bonded layer. A mixed retention mechanism, involving simultaneous interactions (hydrophobic, polar, hydrogen-bond, ion exchange, ...) for either adsorption or partitioning sites, cannot be satisfactorily resolved by linear chromatography, which only measures their sum over all the different types of sites. The high-energy sites are first to be populated at low concentrations, until they are nearly saturated. So, the population of molecules on low-energy sites becomes important only at high solute concentrations. The accurate measurement of the saturation capacities of the different types of sites that coexist in a column and of their corresponding adsorption–desorption constants requires that data be acquired in a concentration range wide enough for the molecular population of each type of sites to vary significantly in some part of the concentration range investigated. Inevitably, measurements at very low and at very high concentrations are necessary to collect sufficient information on the high and the low energy types of sites, respectively. This approach has already been used successfully to derive important information regarding the retention mechanisms occurring in enantioseparations [28–33], to improve our understanding of some particular separations [34,35] and to investigate the thermodynamic behavior of separations on imprinted polymers [36].

In this review, we discuss recent work undertaken to achieve a deeper understanding of the retention mechanism(s) involved in RPLC on modern C_{18}-bonded stationary phases. Our goals are (1) to summarize useful information on the reproducibility of adsorption data that are measured on different packing materials; (2) to report on the degree of heterogeneity of RPLC packing materials; (3) to present new information on mixed retention mechanisms in HPLC; and (4) to illustrate the effects of changing the experimental conditions on the adsorption behavior of neutral and of ionizable compounds. This work presents a battery of new tools that are most effective to compare the performance of different materials and to follow progresses made in the development of new processes for the preparation of better packing materials. If affords new solutions for the prediction and the interpretation of retention data. It summarizes an important amount of experimental results on which new theoretical models of retention in RPLC could be based.

2. Methods used to study adsorption mechanisms

The conclusions that we draw later in this review are the results of an investigation strategy characterized by two important features. The first one consists in concentrating all the necessary efforts needed to measure most accurately single-component adsorption data using a chromatographic method. Admittedly, the data needed could have been acquired by a classical static method. However, these methods are laborious, they require significantly more chemicals than chromatographic methods (except for the mobile phase) and, most importantly, they tend to be poorly accurate and moderately precise. In contrast, the chromatographic dynamic methods have been proven to be highly accurate. Chromatography allows the choice between various approaches and techniques to measure adsorption isotherms. The one mostly used are frontal analysis (FA), frontal analysis by characteristic point (FACP), elution by characteristic points (ECP), the pulse methods, and the computation of elution profiles (CEP) method or inverse method. The advantages and drawbacks of all these methods are discussed in detail elsewhere [37]. The choice of the method used is specific to the problem studied and depends on the type of equipment available, the possible requirement for detector calibration, and the cost and availability of the chemicals studied. When highly accurate adsorption isotherms are needed, FA is by far the most convenient method because it does not require detector calibration (unless mass transfer kinetics is slow) and it does not depend on the column efficiency. This method is described in the next subsection.

The second feature of our approach deals with the handling of the FA adsorption data in order to determine an adsorption isotherm model which is consistent with the whole set of data collected and makes physical sense regarding the chromatographic system studied. Specific representations of the adsorption data (e.g., Scatchard plot), statistical analysis of the data (using e.g., multi-linear regression analysis, the Fisher parameter), calculation of the adsorption energy distribution (using the Expectation-Maximization method) and the comparison of calculated and experimental overloaded band profiles permit the selection of a model, the derivation of the best estimates of its parameters, and the validation of the isotherm obtained. The different tools required to perform these operations successfully are presented in the next few subsections. Finally, there is strength in numbers. The accumulation of many adsorption isotherms, involving numerous compounds (with e.g., different molecular sizes, polarities and polarizabilities, iogenic abilities) and a wide range of experimental conditions (e.g., temperature, pressure, mobile phase composition, nature of the organic modifier) will permit a greater sophistication in our analysis of retention mechanisms in RPLC. They will also supply makers of advanced packing materials with a long needed set of tools useful to understand the consequences of changes made in manufacturing processes.

2.1. The frontal analysis method

This method was originally proposed by James and Phillips [38], and by Schay and Szekely [39] for the determination of adsorption isotherms. Its advantages are discussed elsewhere [37,40]. Its principle consists in replacing abruptly the mobile phase pumped into the column with a solution of the compound studied at a known concentration in the same mobile phase and in recording the composition of the column eluate. The profile recorded is called the breakthrough curve. It is a titration curve since it gives the amount of the compound that is required to equilibrate the packing material in the column with the new solution. The recording lasts as long as necessary to reach thermodynamic equilibrium between the liquid (mobile) and the solid (stationary) phases. This equilibrium is reached when a plateau concentration corresponding to the feed concentration is detected at the column exit.

The detailed study of the consequences of the conservation of the mass of the compound injected into the column between the switch in mobile phase composition at the column entrance and the detection of equilibrium at its exit leads to split the response into four different areas (see Fig. 1 and areas A_1, A_2, A_3, and A_4). The area A_1 represents the mass of the compound that is present in the volume of the mobile phase outside the column, between the mixer point and the detector cell. This volume is called the extra-column volume, V_{ext}. The area A_2 represents the mass of compound that is contained in the volume of the mobile phase inside the chromatographic column or column hold-up volume, V_0. The area A_4 corresponds to the mass of the compound that leaves the column during the time of the experiment. Finally, the area A_3 represents the mass of the compound that is adsorbed on or in the stationary phase inside the column at equilibrium.

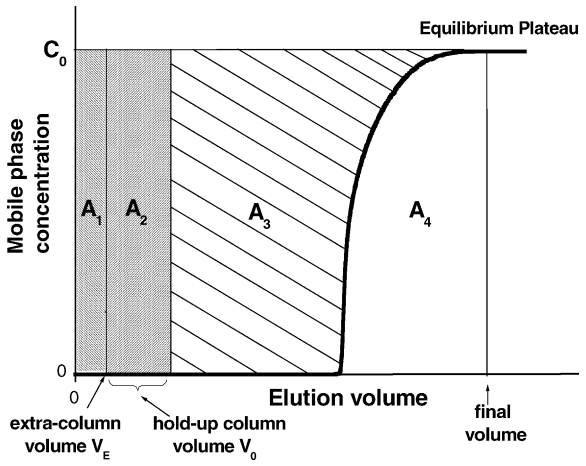


Fig. 1. Frontal analysis method of determination of the equilibrium concentrations in the stationary phase. The breakthrough curve is represented by the thick solid line. The two-hatched surfaces on the left side (A_1 and A_2) represent the mass of compound in the extra and dead column volumes. The area A_3 represent the mass of the compound adsorbed on the surface of the adsorbent. Note that a large error may be made if the area A_3 is determined from the area of a rectangle defined by the inflection point of the breakthrough curves. The equivalent area method is always the best method to measure A_3.

Accordingly, the concentration, q^*_{Vol}, of the component adsorbed (mass adsorbed per unit volume of adsorbent) is:

$$q^*_{Vol} = \frac{A_3}{V_C - V_0} \quad (5)$$

where V_C is the volume of the column tube.

It is noteworthy that the definition of the concentration in the solid phase used in FA is empirical and has no actual physical meaning. Solid adsorbents are porous but are impermeable (with the possible exception of certain resins not considered here). So, solid phase concentrations expressed as the amount of solute adsorbed per unit volume of packing material are not true concentrations in the sense used in thermodynamics, the compound not being distributed homogeneously in a solid phase of a given volume. This unit is merely convenient as its use eliminates the need to measure the specific surface area of the adsorbent. A correct description of interface-related processes, however, should be done by expressing the solid phase concentration in amount of solute adsorbed per unit surface area of the interface. Only the use of such a type of units allows a comparison between the results obtained with different columns. Unfortunately, the determination of the actual surface area of the interface between the liquid phase and the alkyl-bonded packing material is not straightforward because the bonded layer interferes with most methods of measurements. The classical methods of measurements operate under vacuum, collapsing the bonded layer. In the presence of a liquid phase, the layer swells to a degree that depends upon the nature of that solvent. The swelling may obstruct some pores, reducing the actual surface area. It may also be partially permeable to the solute. By convention, the specific surface of the bare silica S_p is taken as the reference surface and this introduces some error. Based on the physico-chemical properties of the packing material (S_p, ρ_{Silica} or density of the bare silica, ρ_{BP} or density of the bonded phase, $\%_{BP}$ or weight

percent of bonded phase), the apparent concentration of solute per unit volume can be transformed into the concentration of adsorbate per unit surface area:

$$q^*_{\text{Surf}} = \frac{q^*_{\text{Vol}} \times ((1 - \%_{\text{BP}})/\rho_{\text{Silica}} + \%_{\text{BP}}/\rho_{\text{BP}})}{S_{\text{p}} \times (1 - \%_{\text{BP}})} \quad (6)$$

In all this paper, we refer to the first definition of q^* per volume unit of stationary phase. If the reader wants to compare the adsorption data reported for different columns, the relevant data in Table 2, which lists the necessary physical properties of the different packing materials, should be introduced into Eq. (6).

In order accurately to determine the concentration of the adsorbate in the stationary phase, an accurate estimate of the column hold-up volume V_0 must be obtained. We found that thiourea is a satisfactory non-retained tracer, despite the fact that it has been reported to be slightly retained [41–43], to a degree that depends on the mobile phase composition. The errors made on the estimate of the column hold-up volume [44] do not affect the profile of the adsorption energy distribution derived from the isotherm data (a bimodal isotherm remains bimodal even if an error of 15% is made on the true hold-up volume). They may affect the value derived for the low-energy equilibrium constant.

The acquisition of FA adsorption data must be made in the broadest possible range of concentrations of the compound. The modeling of the equilibrium isotherms of compounds that are poorly soluble in the mobile phase is less accurate than that of those that are highly soluble. Poor solubility makes compounds ill-suited for systematic studies of retention mechanisms. Isotherm modeling for computer-assisted optimization of the experimental conditions of preparative separations does not suffer from this limitation since measurements of isotherm data can be made at concentrations as high as or higher than those safely used in preparative HPLC and an empirical modeling of the isotherm is acceptable for this purpose. When isotherm data are collected for the purpose of physico-chemical studies (e.g., for the study of retention mechanisms), two rules must be followed to obtain the highest accuracy possible for the adsorption isotherms. First, the maximum concentration in the injected solution, C_{max}, should be as close as possible to the solubility of the component in the mobile phase. This ensures that the largest possible fraction of the surface of the adsorbent will be covered by the compound and permits a more correct estimate of the column saturation capacity. Second, the lowest concentration used for FA measurements must lead to a symmetrical breakthrough curve. Under linear conditions, the shape of the breakthrough curve is an error function, the floating integral function of the classical Gaussian profile. Its position corresponds to the initial slope of the isotherm, i.e., to the Henry constant. Satisfying these two conditions guarantees that the contributions of the highest and lowest-energy types of sites will be taken into account. This is important because the former are occupied at very low concentrations and the latter at high concentrations only.

FA measurements are carried out following a systematic experimental procedure. A mother solution of the compound at a concentration C_{M} in the mobile phase is prepared. Ten adsorption data points are successively acquired by pumping into the column solutions containing 10, 20, ..., 90, and 100% of the mother solution (at C_{M}) in the mobile phase. The mobile phase composition is chosen so that the retention factor of the compound at infinite dilution is between 3 and 5 (accurate measurements require a significantly large breakthrough volume, the need to save on the time and the amount of chemicals needed suggests using a reasonably short breakthrough volume). If the breakthrough curve of the 10% solution is not symmetrical, a new mother solution at $C_{\text{M}}/10$ is prepared and a new series of ten injections is carried out. This procedure is repeated until the two or three breakthrough curves obtained at the lowest concentrations are symmetrical. This might require to change the UV wavelength monitored for detection during the whole set of measurements and to use different wavelengths in the low and high concentration ranges. Experience has shown that, with modern RPLC columns, however low the concentrations below which the isotherm behavior becomes practically linear, the corresponding breakthrough curves can still be detected by the UV detector, the detection limit of which is of the order of 1 μmol/L. We have always been able to measure data in the linear part of the isotherm, except in some rare cases, e.g., for some ionizable compounds eluted with a mobile phase without supporting salts or buffer.

FA measurements can be carried out in two different ways, the staircase and the independent steps methods. In the former method, the column is not regenerated with a stream of the pure mobile phase between two consecutive steps. This method is fast but it is rather inaccurate because errors of measurements propagate from step to step. The estimate of the mass adsorbed during step $i + 1$ depends on the value calculated for the mass adsorbed at the end of the precedent step, i. The latter mode of injection consists in regenerating the column after each step and achieving complete desorption of the compound before injecting a second breakthrough curve. The masses adsorbed during each one of the consecutive steps are calculated independently of the other ones, which eliminates the accumulation of errors. This method requires more time and more chemicals but it is very accurate if the temperature, the back pressure, and the flow rate are well controlled during the entire sequence. The method of independent steps must be preferred for the sake of data accuracy. In addition, recording the complete breakthrough curve (i.e., the adsorption and the desorption profiles for each concentration step injected) has two important advantages. First, it allows the unambiguous determination of the initial linear part of the isotherm, hence informs on the possible need for the acquisition of additional data at lower concentrations. Second, the breakthrough profiles contain important information regarding the mass transfer kinetics.

Fig. 2 illustrates the differences between high and low concentrations breakthrough curves. It shows the breakthrough curves of caffeine (injections during 5 min of solutions at 24.0, 2.64, 0.335, 0.0103 and 0.00103 g/L) on Resolve-C_{18}, using a mixture of methanol and water as the mobile phase (25/75, v/v) [45]. These curves cannot be recorded for concentrations larger than 25 g/L (maximum solubility) but need to be recorded for caffeine concentrations down to about 1 mg/L, i.e., 5 μmol/L. The breakthrough curves become then symmetrical. This means

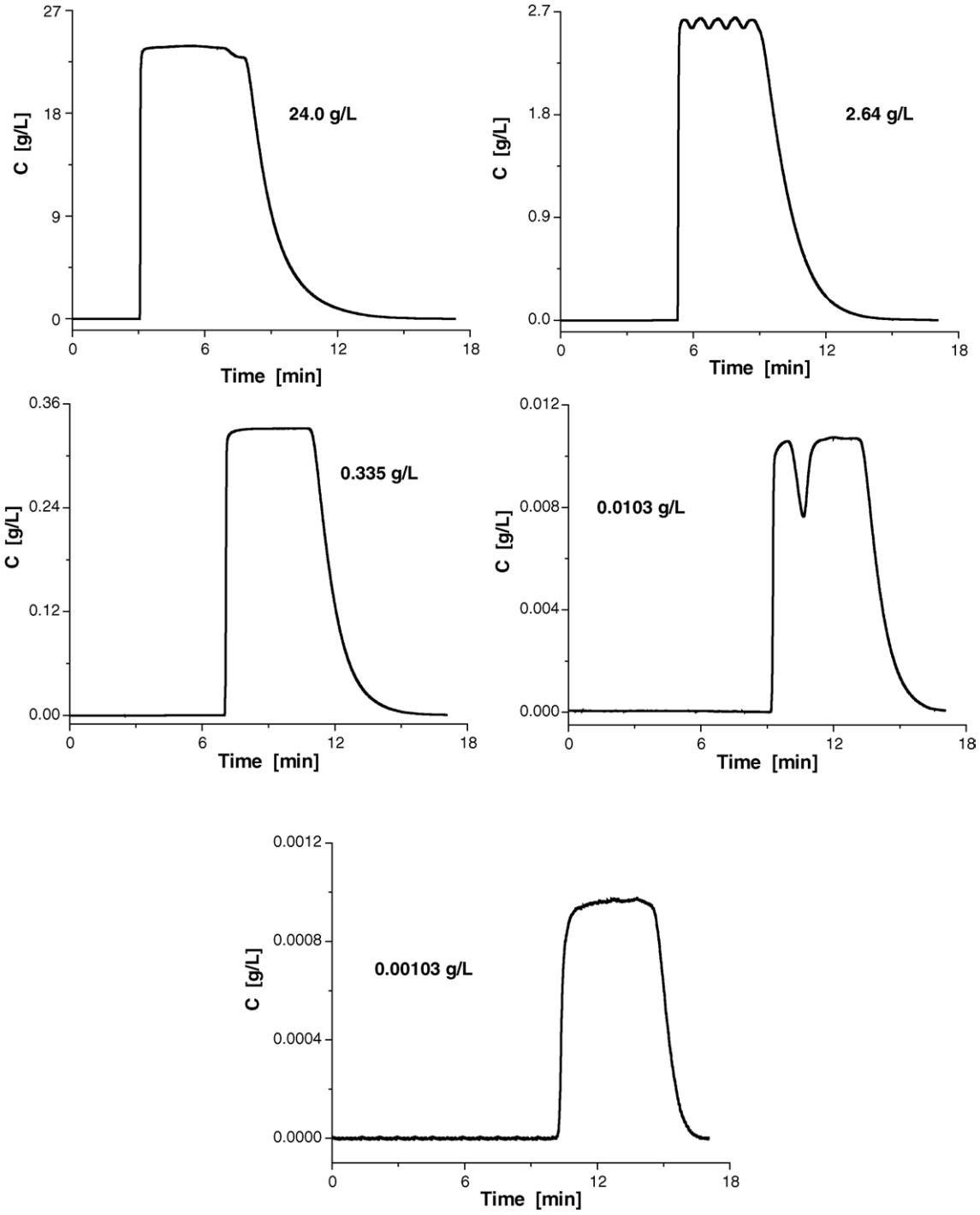


Fig. 2. Example of the evolution of the shape of the breakthrough curves from high concentrations (non-linearity of the isotherm, non symmetric breakthrough curves) to low concentrations (linear domain of the isotherm, symmetric). Caffeine, Resolve-C_{18}, methanol:water, 25/75 (v/v), flow rate 1 mL/min, $T = 295$ K.

that, in this case, the dynamic range of concentrations (i.e., the ratio between the largest and lowest concentrations applied) must be at least 24,000 if one needs the maximum useful information on the adsorption isotherm and wants to optimize the search for the best physical isotherm model.

Measuring adsorption data in a sufficiently wide dynamic range is crucial to identify the different types of adsorption sites that may contribute to the overall retention of the analytes and to determine accurately their parameters (see later, heterogeneity of RP columns). Too small a dynamic range would lead to choose an erroneous models for the fitting of the adsorption data because some isotherm contribution will be missed that varies significantly with the concentration in the range omitted. Fig. 3 shows how important it is to measure adsorption data down to very low concentrations in order accurately to model the presence of very high-energy sites. These sites are usually saturated at very low concentrations. So, beginning the FA run with a concentration in the mobile phase that saturates these sites is the

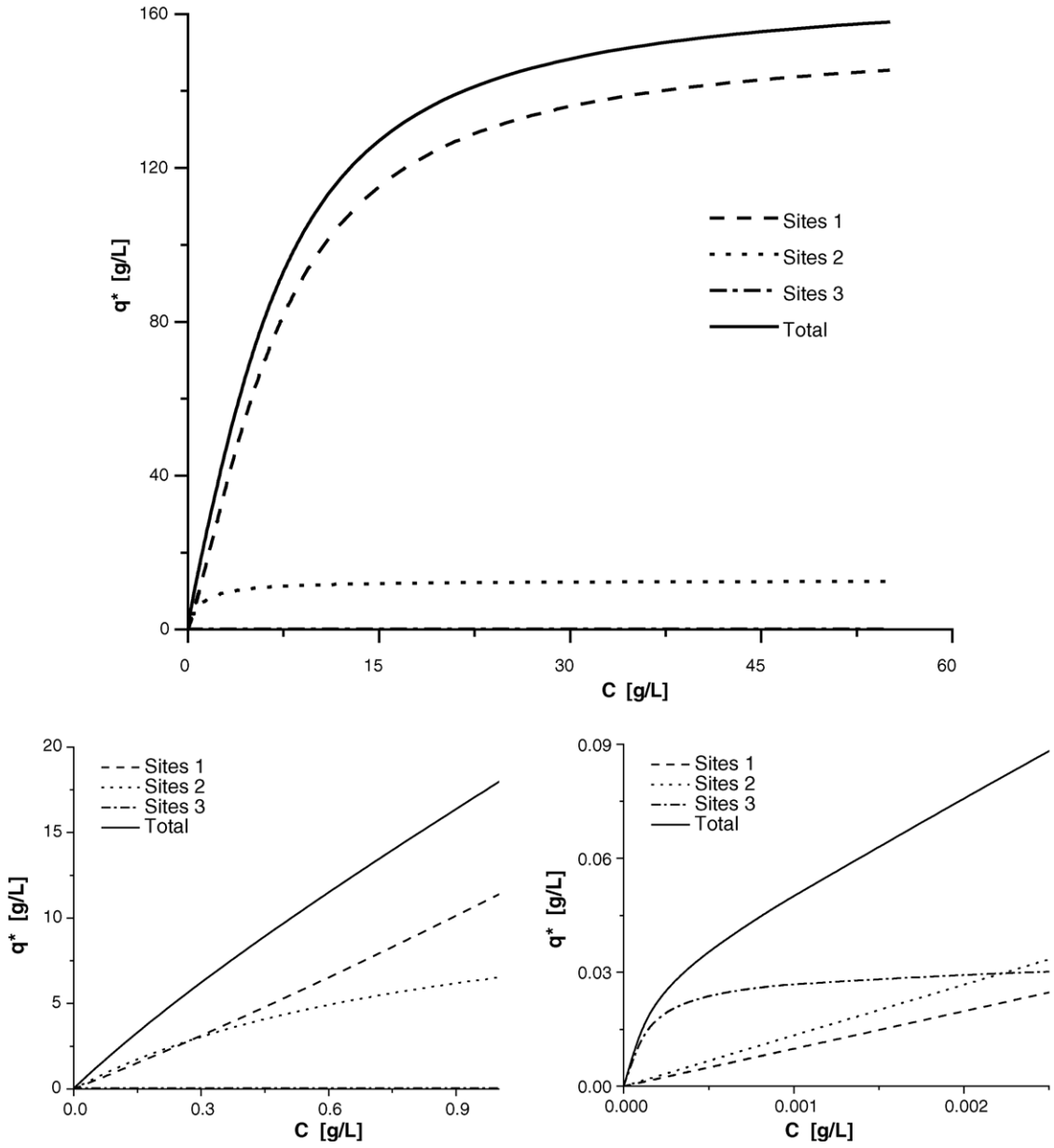


Fig. 3. Plot of the contribution of the three types of adsorption sites identified in the case of Nortryptiline on C_{18}-Discovery column, at $T = 295$ K, with acetonitrile/water, 15/85 (v/v). The figure shows that a dynamic range of at least 60,000 was necessary to describe accurately the adsorption system. Note how different levels of concentration are required to saturate the different adsorption sites, i.e., about 0.001 (bottom-right insert), 7.5, and 60 g/L for sites of types 3–1, respectively. The two bottom inserts show clearly the contribution of adsorption on sites of types 2 and 3, respectively to the overall amount adsorbed in the low concentration range.

most frequent error made in the measurement of FA isotherm data. Even if their number is very small and their contribution to the adsorption of the compound at high concentration is negligible, these sites control to a large extent the retention factor k' that is measurable only under linear conditions (with infinitely diluted solutions). Analysts should not want to miss this precious information.

The aforementioned experimental method and procedures to measure and acquire adsorption data were systematically followed in all the studies presented in this review. The next subsections describe the handling of the FA data in order to derive the best adsorption isotherm model.

2.2. Calculation of the adsorption energy distribution

The issue of the surface heterogeneity of RPLC adsorbents remains confuse for the lack of reliable measurements. The presence of free or unbonded silanol groups on these surface is certain. It results clearly from IR and NMR measurements and from the mass balance between the density of silanol groups on a hydrated silica surface and the number of alkyl chains bonded onto the surface of RPLC materials. However, the accessibility, reactivity, and acidity of these groups is unknown. There are no figures characterizing the difference between the interaction energies of an analyte molecule with a bonded alkyl chain

and with a silanol group. The probabilities of interactions of this molecule and silanol groups or chains are unknown. Obviously, although the alkyl chains are slightly fewer than the silanol groups, the volume occupied by the former is far greater than that of the latter. It is almost certain that interactions between parts of the alkyl chains only and analyte molecules take place often while interactions between these molecules and silanol groups take place almost always in the presence of alkyl groups. For the lack of a suitable method for deriving quantitative estimates of the interaction energy between the adsorbent surface and analyte molecules, we are still content with essentially qualitative tales elaborating inconclusively on the obnoxious presence of underivatized, isolated silanol groups that would be the main if not the only source of heterogeneity of the surface of C_{18} bonded stationary phases. Manufacturers have invested considerable resources in attempts to reduce as much as possible the density of these undesirable silanol groups, using for that purpose new bonding reagents, silanol endcapping, new, bare solid adsorbents with fewer silicon atoms in their network, or adsorbents encapsulated in a polymeric shell, hence, in all cases, with fewer surface silanols. Notable improvements have been made during the last two decades. Still, as we show later, the adsorbents available are far from being reasonably homogeneous.

We know that all surfaces are heterogeneous [46]. This fact is particularly vexing in adsorption studies. It is due to the superimposition of two properties of matters. First, the solubility of impurities in solids tends to be poor and impurities are expelled toward the surface of the solid. The concentrations of all the impurities are always much higher at the surface than in the bulk solid. These impurities cause a strong heterogeneity of the electrostatic field at the silica/gas or silica/liquid interface. This is why the decrease in the iron, boron, or aluminum content of silica that is a consequence of the development of the sol-gel processes for silica synthesis has led to greatly improved silica adsorbents. Second, the very presence of the surface causes stresses and strains of the bonds of polyvalent atoms. Thus, because they are stressed and strained, the valences of the silicon and oxygen atoms at the silica surface are not fully saturated. This also causes a certain degree of heterogeneity of the electrostatic field above the surface, but to a lesser degree. The study of adsorption onto heterogeneous surfaces has become of great interest in adsorption studies.

Actual surfaces are characterized by an adsorption energy distribution function, $F(\epsilon)$, that may be broader or narrower, or that may have several more or less well resolved modes, each mode having a finite width. The experimental isotherm on such a surface is the sum of the isotherm contributions of each one of the different types of homogeneous sites that are covering the surface. Under the condition of a continuous adsorption energy distribution and assuming a Langmuir local isotherm model on each homogeneous patch of the surface, the experimental isotherm can be written [46]:

$$q^*(C) = \int_0^\infty F(\epsilon)\frac{b(\epsilon)C}{1+b(\epsilon)C}\mathrm{d}\epsilon \tag{7}$$

where $q^*(C)$ is the total amount of a solute adsorbed on the surface at equilibrium with a concentration C of this solute, ϵ the binding energy between an adsorbed solute molecule and the surface of the adsorbent, and b is the associated binding constant related to ϵ through the following equation:

$$b(\epsilon) = b_0 \exp\left(\frac{\epsilon}{RT}\right) \tag{8}$$

where b_0 is a pre-exponential factor that is usually assumed to be the same whatever the type of adsorption sites, i, that is considered [46].

In Eq. (7), other local isotherm models can be considered. The most important are the Jovanovic isotherm [47],

$$1 - \exp(-b(\epsilon)C) \tag{9}$$

the Moreau isotherm [48],

$$\frac{b(\epsilon)C + Ib(\epsilon)^2C^2}{1 + 2b(\epsilon)C + Ib(\epsilon)^2C^2} \tag{10}$$

which is valid for nonideal adsorption on a homogeneous surface, i.e., for adsorption under such conditions that significant adsorbate–adsorbate interactions may take place and in which I is the adsorbate–adsorbate isotherm parameter, or the BET isotherm [49]

$$\frac{b(\epsilon)C}{(1 - b_L(\epsilon)C)(1 - b_L(\epsilon)C + b(\epsilon)C)} \tag{11}$$

where b_L is the equilibrium constant of the component between successive layers of adsorbate and the mobile phase. The difficulty to solve Eq. (7) and obtain the energy distribution with the Moreau or the BET isotherm is due to the presence of a second independent parameter (the adsorbate–adsorbate interaction coefficient, I or b_L) in addition to the equilibrium constant b. This two-dimension problem has not been solved yet and the only possibility to derive $F(\epsilon)$ in this case would consist in assuming a relationship between $b(\epsilon)$ and I or b_L. It could make sense to propose that the higher the equilibrium constant $b(\epsilon)$, the lower the adsorbate–adsorbate interactions because the distance between two closest adsorption sites would then be larger. However, any formal relationship would be empirical at this stage.

In many cases of liquid/solid adsorption, however, we found that the adsorbate–adsorbate interactions are negligible and that the local adsorption behavior is correctly accounted for by the Langmuir model. In this work, we discuss only cases in which the local isotherm follows this model and the problem is only to find the corresponding distribution function, a function of the equilibrium constant, $b(\epsilon)$, alone.

The normalization condition for the AED is a boundary condition for Eq. (7)

$$\int_0^\infty F(\epsilon)\,\mathrm{d}\epsilon = q_s \tag{12}$$

where q_s is the overall saturation capacity.

To characterize the behavior of a heterogeneous surface, the AED, $F(\epsilon)$, is derived from the set of experimental isotherm data (with the M experimental data points given by $M - 1$ FA measurements plus the origin, $q = C = 0$), a procedure for which

there is a variety of methods [46,50–52]. Most of these methods use a preliminary smoothing of the experimental data, i.e., fit these data to an isotherm model (e.g., to the Freundlich isotherm [50]), or search for an AED that is given by an arbitrary functional relationship which is chosen depending on the problem studied and the instinct of the scientist. These two approaches are equivalent because there is a direct correspondence between isotherm and AED models. In both types of methods, arbitrary information is injected into the determination of the AED by forcing it to follow a given functional relationship. In this work, we used instead a numerical method, the EM method [52]. This computer-intensive method uses directly the raw experimental data, without injecting any arbitrary information into the AED derivation. The distribution function, $F(\epsilon)$, is discretized, using an N-grid points in the energy space, (i.e., assuming that the surface is tiled with a set of N different homogeneous surfaces) and the corresponding values of $F(\epsilon)$ are estimated from the M experimental data points. The energy space is limited by $\epsilon_{\min}$ and $\epsilon_{\max}$, two energy boundaries which are respectively related to the maximum and the minimum concentrations within which the adsorption data have been acquired, by using Eq. (8) (with $b_{\min} = 1/C_M$ and $b_{\max} = 1/C_1$). This is why FA data must be acquired in as wide a concentration range as possible. However, a narrower range may be considered, as long as it accommodates the data.

The amount $q(C_j)$ of solute adsorbed at concentration C_j is iteratively estimated by

$$q^k_{\text{cal}}(C_j) = \sum_{\epsilon_{\min}}^{\epsilon_{\max}} F^k(\epsilon_i) \frac{b(\epsilon_i)C_j}{1 + b(\epsilon_i)C_j} \Delta\epsilon, \quad j \in [1, M]; i \in [1, N] \tag{13}$$

with

$$\Delta\epsilon = \frac{\epsilon_{\max} - \epsilon_{\min}}{N - 1}, \qquad \epsilon_i = \epsilon_{\min} + (i - 1)\Delta\epsilon \tag{14}$$

The index k indicates the kth iteration of the numerical calculation of the AED function. The initial guess (iteration $k = 0$) of the AED function, $F(\epsilon_i)$, is the uniform distribution (over the N fictitious adsorption sites) of the maximum adsorbed amount that was observed experimentally [52].

$$F^0(\epsilon_i) = \frac{q(C_M)}{N}, \qquad \forall i \in [1, N] \tag{15}$$

Using this initial guess has the advantage of introducing the minimum possible bias into the AED calculation. Actually, the EM program calculates the amount adsorbed by taking $b(\epsilon_i)$ as the variable in the energy space, so that neither the temperature nor the pre-exponential factor in Eq. (8) need to be defined [52]. Only M, N, $b_{\min}$, $b_{\max}$ and the number of iterations must be defined before starting the calculations. It is noteworthy, however, that, while an assumption must be made regarding the value of b_0 in Eq. (8) in order to obtain any information on the adsorption energy, this assumption cancels out when the difference between two adsorption energies is calculated (see below). The final result is the distribution of the equilibrium constants (often called the affinity distribution). The distribution function is updated after each iteration by

$$F^{k+1}(\epsilon_i) = F^k(\epsilon_i) \sum_{C_{\min}}^{C_{\max}} \frac{b(\epsilon_i)C_j}{1 + b(\epsilon_i)C_j} \Delta\epsilon \frac{q_{\exp}(C_j)}{q^k_{\text{cal}}(C_j)} \tag{16}$$

The EM procedure protects better than most other methods against the consequences of the possible incorporation of experimental artifacts into the calculation of the AED and against the effect of modeling the experimental data (and particularly the noise and drift that the data may contain) [52].

Finally, we obtain the AED or relationship between the adsorption constant (b_i) on a type of sites (i) and the number of adsorption sites ($q_{S,i}$) over which the compound is distributed at equilibrium between the liquid and the adsorbent. This AED ($q_{S,i}(b_i)$), with $b_i = b(\epsilon_i)$ is considered as valid for further investigations if $q_{S,i}$ tends towards zero when b_i tends towards $b_{\min}$ and $b_{\max}$. If it does not do so, the number of iterations should be increased until the following criteria is fulfilled:

$$\frac{q_{S,1}}{\text{Max} q_{S,i}} < 0.01 \qquad \text{and} \qquad \frac{q_{S,N}}{\text{Max} q_{S,i}} < 0.01 \tag{17}$$

If these conditions cannot be realized for any iteration number superior to one hundred millions, this usually means that the maximum experimental concentration $C_{\max} = C_M$ at which FA measurements were carried out is too low to get a good estimate of the properties of low-energy sites by the EM calculation. The maximum surface coverage achieved is definitely too low. This limitation comes usually from the poor solubility of the compound studied. Rarely, another limit is encountered when the minimum concentration at which FA data can be acquired is not low enough, which may come from a lack of detector sensitivity and then, the divergence of the AED is observed in the high adsorption constant range. In either case, the AED obtained can provide only a qualitative answer to the question of the degree of heterogeneity of the adsorbent studied. Only the number of distinct sites can be obtained, not precise estimates of the saturation capacities and average equilibrium constants of these sites.

2.3. The equilibrium dispersive model

Overloaded elution band profiles or breakthrough curves can be calculated using the best model derived (see later section) for the isotherm of the compound studied and the equilibrium dispersive model (ED) of chromatography [37,53,54]. The ED model assumes instantaneous equilibrium between the mobile and the stationary phases and a finite column efficiency originating from an apparent axial dispersion coefficient, D_a, that accounts for the dispersive phenomena (molecular and eddy diffusion) and also for the non-equilibrium effects that take place in a chromatographic column. These effects are supposed to be relatively small compared to the band broadening originating from the nonlinear behavior of the isotherm, otherwise the ED model would not be valid. The axial dispersion coefficient is related to the experimental parameters through the following equation:

$$D_a = \frac{uL}{2N} \tag{18}$$

where u is the mobile phase linear velocity, L the column length, and N is the number of theoretical plates or apparent efficiency of the column. In the ED model, the mass balance equation for a single component is written

$$\frac{\partial C}{\partial t} + u\frac{\partial C}{\partial z} + F\frac{\partial q^*}{\partial t} = D_a\frac{\partial^2 C}{\partial z^2} \quad (19)$$

where q^* and C are the stationary and the mobile phase concentrations of the adsorbate, respectively, t the time, z the distance along the column, and $F = (1-\epsilon_t)/\epsilon_t$ is the phase ratio, with ϵ_t the total column porosity at time t and distance z. If ϵ_t is assumed to be constant during the whole measurement, so is F (in some cases, particularly when the BET model of isotherm applies, F is a function of the local value of q^*, hence of C and consequently it is a function of *tandz*). q^* is related to C through the isotherm equation, $q^* = f(C)$.

2.3.1. Initial and boundary conditions for the ED model

At $t = 0$, the concentrations of the solute and the adsorbate in the column are uniformly equal to zero, the column is empty of solute, and the stationary phase is in equilibrium with a stream of the pure mobile phase. The boundary conditions used are the classical Danckwerts-type boundary conditions [37,55] at the inlet and the outlet of the column.

2.3.2. Numerical solutions of the ED model

The ED model was solved using the Rouchon program based on the finite difference method [37,56–58].

2.4. From the isotherm data to the isotherm model

Once the single-component adsorption data have been collected, they need to be properly modeled. The main isotherm models available are discussed in the next section. The following tests are applied successively to find out whether an isotherm model is consistent with the experimental data, which models are acceptable, and which models have to be definitely rejected. Thus, the selection of the best isotherm model obeys to a process of elimination until one single isotherm model is left.

- The first test consists in plotting the data as a Scatchard plot (e.g., a plot of q^*/C versus q^*. Isotherm models can be classified according to the shape of the corresponding Scatchard plot. As illustrated in Fig. 4, some isotherms have a convex downward Scatchard plot (e.g., the bi-Langmuir, the Tóth, the Langmuir-Freundlich models), others have a convex upward Scatchard plot (e.g., the Jovanovic, the Fowler models). Isotherm models with an inflection point have a Scatchard plot with a minimum, a maximum, or both. Only the Langmuir isotherm model has a linear Scatchard plot. The shape of the experimental Scatchard plot informs on the group of models to consider.
- The second step of our selection process consists in modeling the isotherm data with those in the selected group of models. A multi linear regression analysis of these models is carried out, using a fitting based on the Marquardt algorithm [59], which minimizes the residual sum of the squares of the relative differences between the experimental data and those calculated with the model. The statistical Fisher parameter, F_t, is used to select the best isotherm model. For each model, the Fisher parameter is calculated according to:

$$F_{\mathrm{calc},t} = \frac{N-l}{N-1}\frac{\sum_{i=1}^{i=N}(q_{\mathrm{exp},i} - \overline{q_{\mathrm{exp}}})^2}{\sum_{i=1}^{i=N}(q_{\mathrm{exp},i} - q_{t,i})^2} \quad (20)$$

where $q_{\mathrm{exp},i}$ are the experimental values of the solid phase concentrations of the adsorbate in equilibrium with a liquid phase at concentrations C_i (i.e., $q_{\mathrm{exp},i}$ and C_i are the FA data points), $\overline{q_{\mathrm{exp}}}$ the mean value of the whole set of data, $q_{\mathrm{exp},i}$, $q_{t,i}$ the estimate given by the isotherm model ($\mathcal{M}_t$) for the solid phase concentration of the adsorbate in equilibrium with the mobile phase concentration C_i, l the number of adjusted parameters in the model, and N is the number of experimental data acquired by FA.

In order to tell if a model is significantly (or statistically) better than another one, the F-test ratio between two models $\mathcal{M}_{t_1}$ and $\mathcal{M}_{t_2}$, F_{t_1,t_2} is calculated by:

$$F_{t_1,t_2} = \frac{F_{t_1}}{F_{t_2}} \quad (21)$$

Considering a risk α, the model $\mathcal{M}_{t_1}$ correlates better the experimental data than the model $\mathcal{M}_{t_2}$ if

$$F_{t_1,t_2} \geq F_{N-l_1,N-l_2,\alpha} \quad (22)$$

where l_1 and l_2 are the numbers of adjusted parameters in the models $\mathcal{M}_{t_1}$ and $\mathcal{M}_{t_2}$, respectively. $F_{N-l_1,N-l_2,\alpha}$ is available in statistical test tables. If, for a given model, $\mathcal{M}_{t_2}$, Eq. (22) is valid whatever the model $\mathcal{M}_{t_1}$, then the model $\mathcal{M}_{t_2}$ will be definitively eliminated.
- The third step compares the set of remaining isotherm models with the results of the calculation of the adsorption energy distributions from the raw adsorption data (see Section 2.2). Any adsorption model that is inconsistent with the experimental AED must be eliminated. For instance, if the EM calculations converge toward a bimodal distribution, a Tóth model cannot account properly for the isotherm data since the AED of the Tóth model is unimodal.
- Finally, if after the third step, there are still more than one isotherm model that may account correctly for the adsorption data, the last selection step is based on the comparison between the calculated and the experimental band profiles at high and low concentrations. Still, if no significative difference is found between these models, the final choice will be based on the physical sense of the isotherm parameters.

3. Adsorption isotherm models

For the last two decades most of the adsorption isotherms measured in RPLC were found langmuirian, i.e., they were found to be convex upward and to belong to type I of the gas–solid equilibrium isotherm classification of Brunauer et al. [60]. Very few cases had shown adsorption data consistent with isotherms of type II (S-shaped isotherms that are convex upward at low concentrations then convex downward at high concentrations),

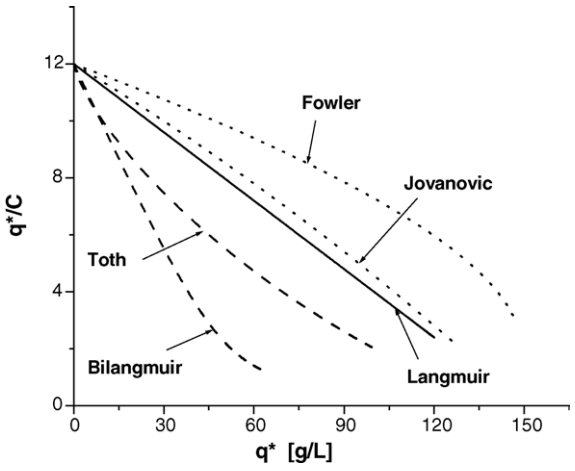


Fig. 4. Correspondance between the shape of the Scatchard plot representation (q^*/C vs. q^*) and the nature of the isotherm model. Reproduced with permission from Gritti and Guiochon [78] (Fig. 1).

to type V (S-shaped isotherm that are convex downward at low concentrations then convex upward at high concentrations) or to type III (antilangmuirian isotherms that are convex downward). Recent investigations, however, have demonstrated that all types of isotherm shape could be observed in RPLC. This seems to be due in part to the wider range of structure and physico-chemical properties of the compounds studied, in part to the understanding that isotherms must be measured in the widest concentration range possible.

The accumulation of a large amount of experimental data has made possible to propose some qualitative predictive rules relating the shape of the isotherm and the characteristics of the solute studied. Simple conclusions can be drawn from these experimental results regarding the mechanisms of adsorption in RPLC, as we show later in this report.

3.1. Nature of the solute and curvature of the adsorption isotherm

Table 1 lists 15 compounds for which isotherms were measured on one or several of the RPLC columns studied (using methanol:water solutions as the mobile phase), together with some physico-chemical characteristics of these compounds and the shape of the isotherms measured for them. A solute can be characterized by a few molecular descriptors. Abraham et al. have published a list of more than 400 solutes with the values of numerous of these descriptors [61]. The descriptors considered measure the propensity of a solute (1) to interact with a solvent through Π- or n-electron pairs (descriptor R_2); (2) to take part in dipole–dipole or dipole-induced interactions (descriptor π_2^H); (3) to release an hydrogen atom to form hydrogen bonds with the solvent (descriptor $\sum \alpha_2^H$); (4) conversely, to accept hydrogen atoms from the solvent and form hydrogen bonds (descriptor $\sum \beta_2^H$); (5) a last descriptor, the van der Waals volume V_x of the solute, is related both to the energy required to create a cavity to accommodate the solute in the solvent and to the dispersion interactions between the solute and the solvent.

Three out of these five descriptors are statistically independent for the family of solutes studied. For this reason, only the hydrogen bond acidity ($\sum \alpha_2^H$), the hydrogen bond basicity ($\sum \beta_2^H$), and the solute hydrophobicity (V_x) are shown in Table 1. Although relatively restricted, the number of solutes is sufficient to use their descriptors in an attempt at characterizing, on a statistical basis, the physico-chemical properties of chromatographic systems depending on the nature of the stationary phase and of the mobile phase used. This will shed light on the relative importance of the interactions that govern the retention mechanisms.

In all our experiments, the methanol content of the mobile phase was always adjusted in order to achieve a moderate value of the retention factor. Too short a retention factor gives poorly accurate FA data. Too long a retention time of the solute leads to a waste of time and chemicals. Typically, the mobile phase composition was adjusted to achieve retention factors k_0' at infinite dilution of around 5. Table 1 shows a wide range of methanol content of the mobile phase, from 10 to 80% for the low-molecular-mass compounds used. Fig. 5 shows plots of the values of the three main molecular descriptors versus the methanol content of the mobile phase. No useful correlation can be established between the hydrogen-bond acidity, the hydrogen-bond basicity, or the hydrophobicity of the solute and the methanol content required

Table 1
Molecular descriptors of 15 analytes, isotherm shape and content of methanol in the aqueous mobile phase

	π_2^H	$\sum \alpha_2^H$	$\sum \beta_2^H$	V_x	% MeOH (v)	Isotherm shape	References
3-Phenyl 1-propanol	0.91	0.30	0.65	1.06	50	Langmuir	[63]
4-*tert*-Butylphenol	0.89	0.56	0.39	1.34	60–62	Langmuir	[63,135]
Butylbenzene	0.49	0	0.17	1.14	80	Anti-Langmuir	[63]
Butylbenzoate	0.85	0	0.46	1.50	60–80	S-shaped	[63,136]
Phenol	0.89	0.60	0.31	0.78	15–45	Langmuir	[69,79,85,77,78,45,105,96]
Caffeine	2.69	0	1.80	1.50	15–30	Langmuir	[69,79,77,78,45,135,72]
Toluene	0.52	0	0.14	0.86	80	S-shaped	[35]
Ethylbenzene	0.51	0	0.15	1.00	80	S-shaped	[35]
Aniline	0.89	0.23	0.45	0.82	15–45	Langmuir S-shaped	[74,77]
Theophylline	2.54	0	1.80	1.36	30	Langmuir	[74]
Propranolol	1.71	0.45	1.53	2.22	25–40	S-shaped	[74,122,128–136]
Ethylbenzoate	0.85	0	0.46	1.21	62	S-shaped	[64–66]
Propylbenzoate	0.85	0	0.46	1.35	60–65	S-shaped	[77,135]
1-Naphthalene sufonate	2.92	0	2.84	1.69	10	Langmuir	[132]
Amylbenzene	0.48	0	0.18	1.28	80	Anti-Langmuir	[62]

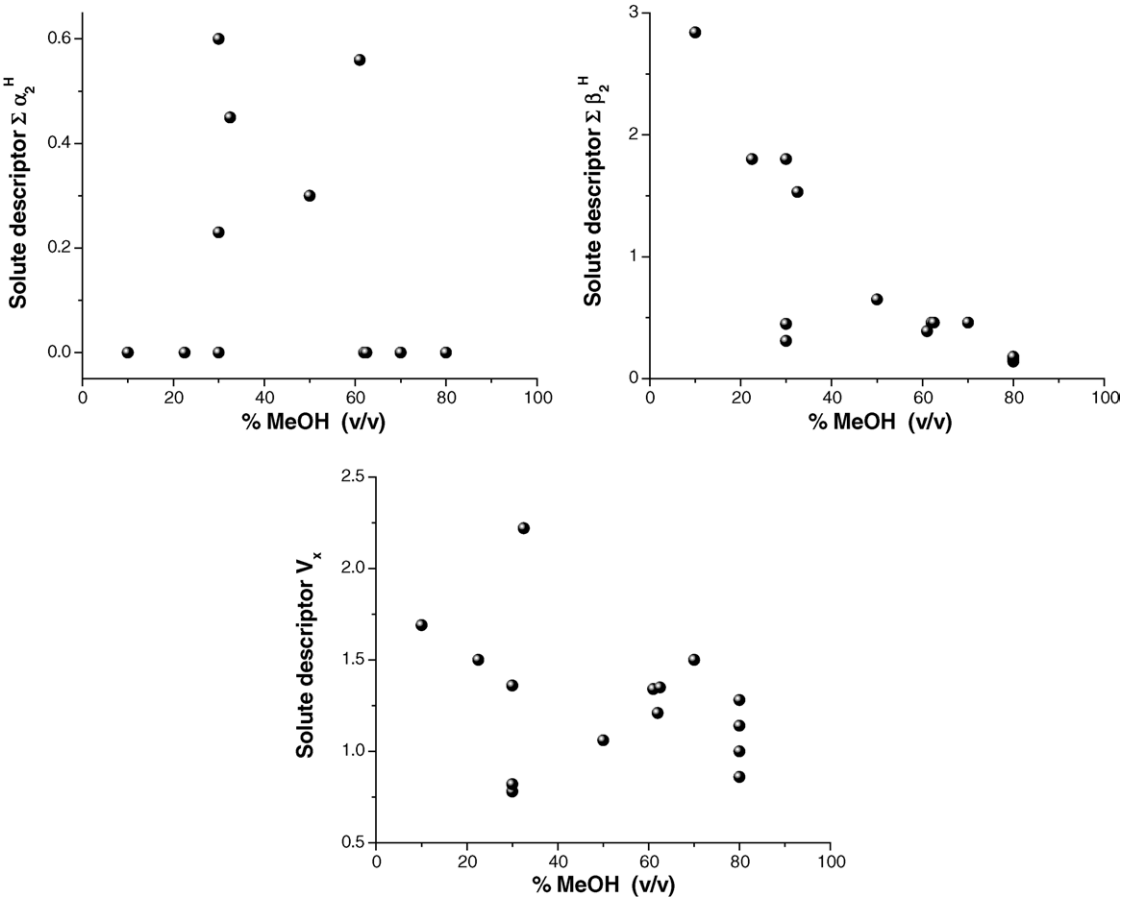


Fig. 5. Plots of the molecular descriptors of the analyte (hydrogen bond acidity $\sum \alpha_2^H$, hydrogen bond basicity $\sum \beta_2^H$ and molecular volume V_x) vs. the content of organic modifier (methanol) required to measure accurate adsorption data ($k' > 3$) in RP-HPLC conditions. Note the poor correlation between these individual descriptors and the methanol content.

to achieve a retention factor close to 5. Note, however, that we never needed to use a high methanol content mobile phase to elute a solute with a strong tendency to form hydrogen bonds by accepting hydrogen atoms. More generally, it is clear that none of these parameters control the retention of the solute in RPLC with aqueous methanol solutions. Instead, a combination of all these parameters must be considered. Qualitatively, for entropic reasons, the larger the molecular volume of a solute, the more likely its expulsion from the polar liquid phase. On the other hand, the stronger the ability of the solute to participate in hydrogen bonding interactions with the solvent molecules, the better its affinity for methanol and water. Accordingly, V_x, on the one hand, $\sum \alpha_2^H$ and $\sum \beta_2^H$, on the other act in opposite directions. A more appropriate solute descriptor to correlate the methanol content of the mobile phase and its retention factor would be $P = V_x - \sum \alpha_2^H - \sum \beta_2^H$. Fig. 6 shows that there is indeed a clear correlation between P and the methanol concentration in the mobile phase.

Most interesting is the correlation between the location of the points corresponding to the different compounds studied and the shape of the isotherm measured. Antilangmuirian isotherms are observed for high values of P and high methanol contents, corresponding to apolar solutes (e.g., amyl benzene, butyl benzene [62]). Langmuirian isotherms are found in the region of low values of P and low methanol concentrations, corresponding to polar, hydrogen-bond donor or acceptor solutes (e.g., caffeine, phenol). Finally, the intermediate region of moderate values of P and of the methanol concentration contains the points repre-

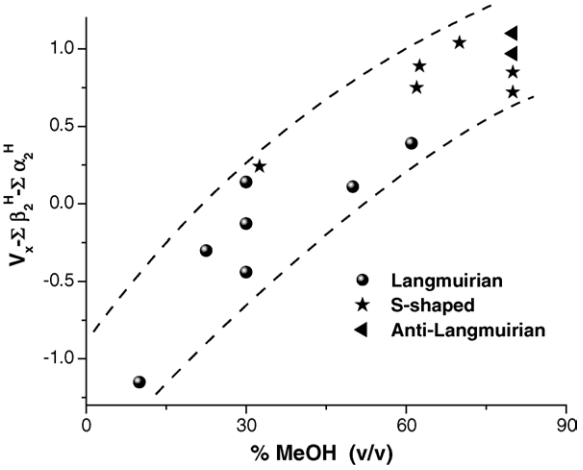


Fig. 6. Same as in Fig. 5, except for the plot of $V_x - \sum \alpha_2^H - \sum \beta_2^H$ vs. the methanol content. Note, this time, a correlation between this new built descriptor, the quantity of methanol and the nature of the isotherm (Langmuir, S-shaped, anti-Langmuir).

senting the compounds that have S-shaped isotherms (e.g. esters like propyl benzoate or small apolar solutes like toluene).

This is illustrated by the comparison between the adsorption isotherms of 4-*tert*-butyl phenol ($P = 0.39$, [MeOH] = 61%), ethyl benzoate ($P = 0.75$, [MeOH] = 62%) and amyl benzene ($P = 1.10$, [MeOH] = 80%). These three compounds have an aromatic ring, nearly the same molecular weight (150, 150, and 148 g/mol, respectively), the same van der Waals volume ($V_x = 1.34$, 1.21, and 1.28, respectively) but have adsorption isotherms of types I–III, respectively, on all the C_{18} bonded phases tested. Their different behavior is due to their different ability at participating in hydrogen-bond interactions with the mobile phase. While 4-*tert*-butyl phenol is both an acceptor and a donor of hydrogen, ethyl benzoate is merely an hydrogen bond acceptor, and amyl benzene is a very poor hydrogen bond acceptor. The modeling of the adsorption data of these three compounds [63–66] has proven that the adsorption isotherm of 4-*tert*-butyl phenol follows Langmuir behavior, hence is simply controlled by one equilibrium constant, b, which accounts for the equilibrium between the two states of the compound, solute and adsorbate. In contrast, the adsorption of the other two components is characterized by two independent equilibrium constants, b_S and b_L. The first accounts for the equilibrium of the compound between the solution and the bare C_{18}-bonded surface (b_S), the second for the equilibrium between the solution and the successive adsorbed layers of compound (b_L). In other words, molecules of 4-*tert*-butyl phenol do not experience adsorbate–adsorbate interactions while those of ethyl benzoate and amyl benzene do.

A schematic representation of these adsorption mechanisms that is consistent with these adsorption models is given in Figs. 7–9. In the case of 4-*tert*-butyl phenol, the adsorbed phase

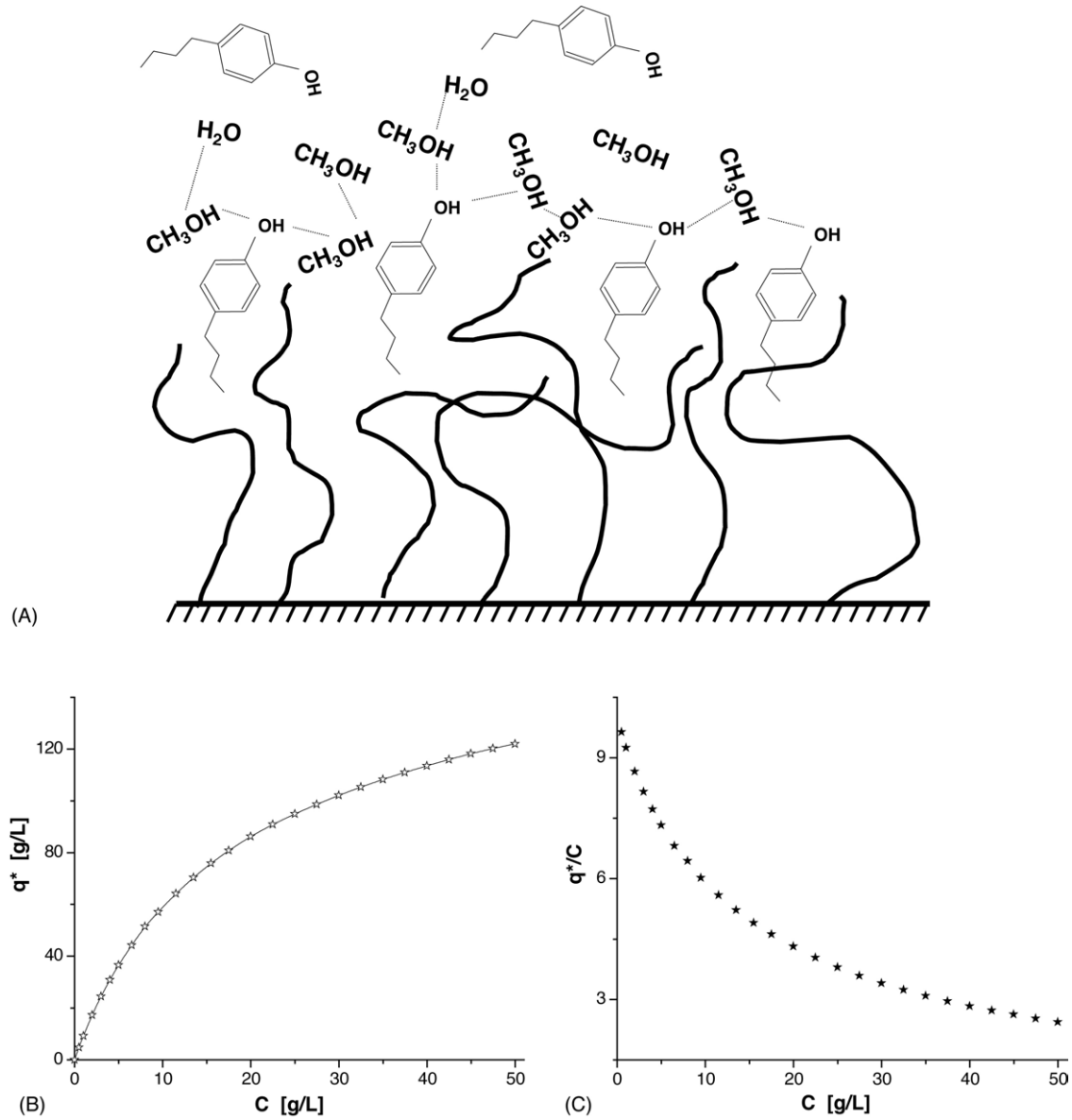


Fig. 7. Scheme (A) of the molecular description of the adsorption of analytes having a langmuirian isotherm (B and C). The adsorption is limited to a monolayer of adsorbate molecules.

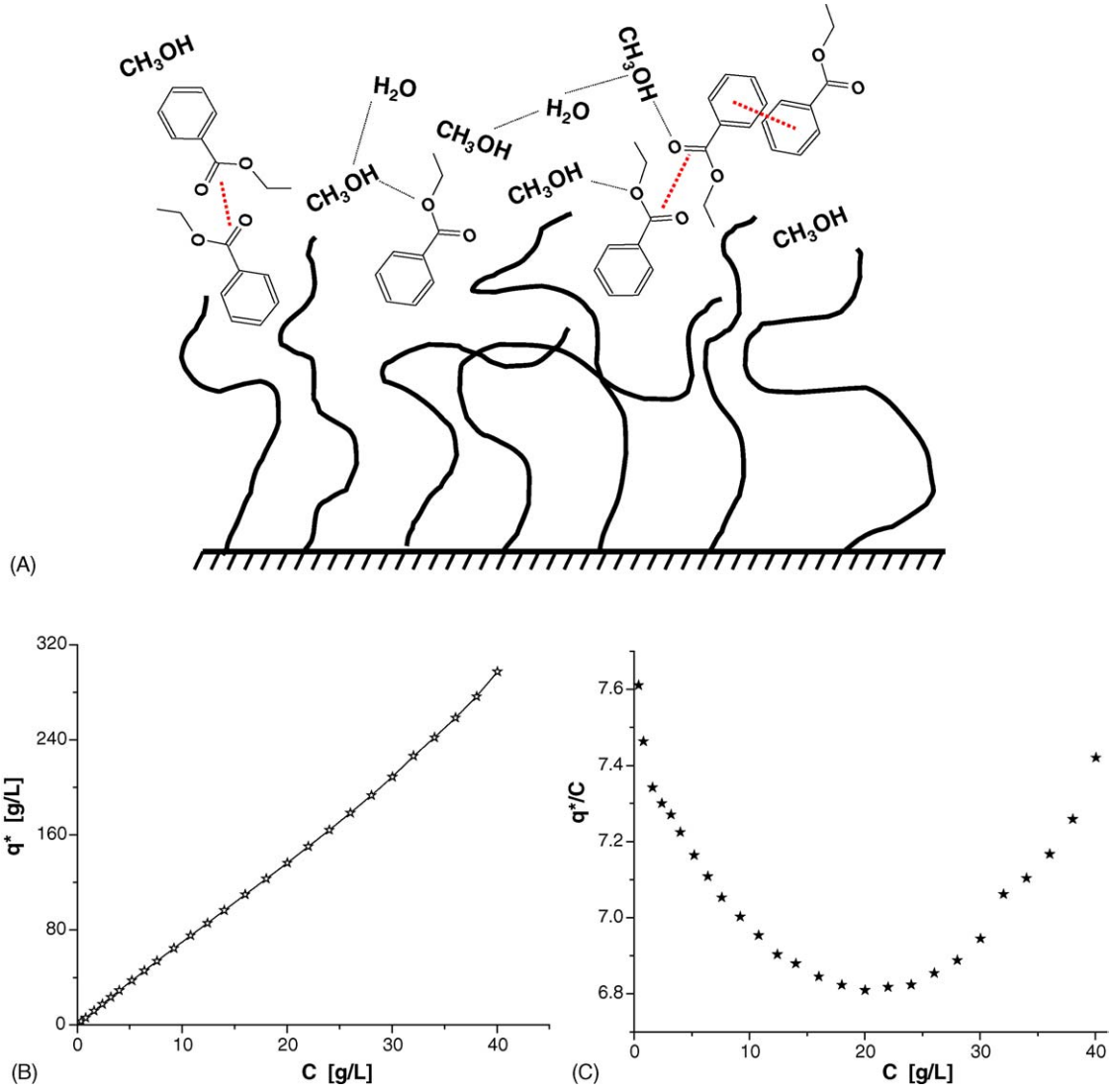


Fig. 8. Same as in Fig. 7, except for analytes whose isotherm is described by a S-shaped adsorption isotherm. Adsorbate–adsorbate interactions occur and become predominant at high concentrations.

is limited to a monolayer because the hydrophobic part of its molecules interacts within the tips of the C_{18} chains while the polar part is solvated with adsorbed methanol molecules, through strong hydrogen-bonds. For ethyl benzoate, the situation is different because the solvation of the polar part (ester group) by methanol molecules is weaker (the solute can only receive hydrogen bonding from methanol, not give such bonding). This makes it more likely that molecules of ethyl benzoate interact together in the adsorbed state, through dipole–dipole interactions involving the carbonyl group or Π-stacking between phenyl rings. Even though it is not possible to ascertain the actual structure of the adsorbed phase, the formation of adsorbate–adsorbate interactions at high concentrations is made clear by the S-shaped isotherm of ethyl benzoate. The antilangmuirian shape of the adsorption isotherm of amyl benzene shows that adsorbate–adsorbate interactions take place even at zero concentration in the mobile phase. Because amyl benzene molecules cannot favorably interact with polar solvents like methanol, there is no competition to adsorbate–adsorbate interactions. Most of the adsorbed methanol is displaced from the interface by the analyte which interacts more strongly with the bonded alkyl chains.

This qualitative molecular description of the adsorbed phase is based on the well-known nature of the interactions between the analyte and the organic solvent (methanol). It is consistent with the adsorption data measured and, particularly, with the shape of the equilibrium isotherms observed.

3.2. Mathematical models

Liquid/solid equilibrium isotherms can be separated into four classes, those corresponding to ideal adsorption (no adsorbate–adsorbate interactions) on homogeneous surface (with an AED given by a Dirac δ function or a narrow Gaussian curve, to account for experimental errors and for small fluctuations of the surface properties caused e.g., by its roughness), to ideal adsorption on heterogeneous surfaces, to nonideal adsorption on

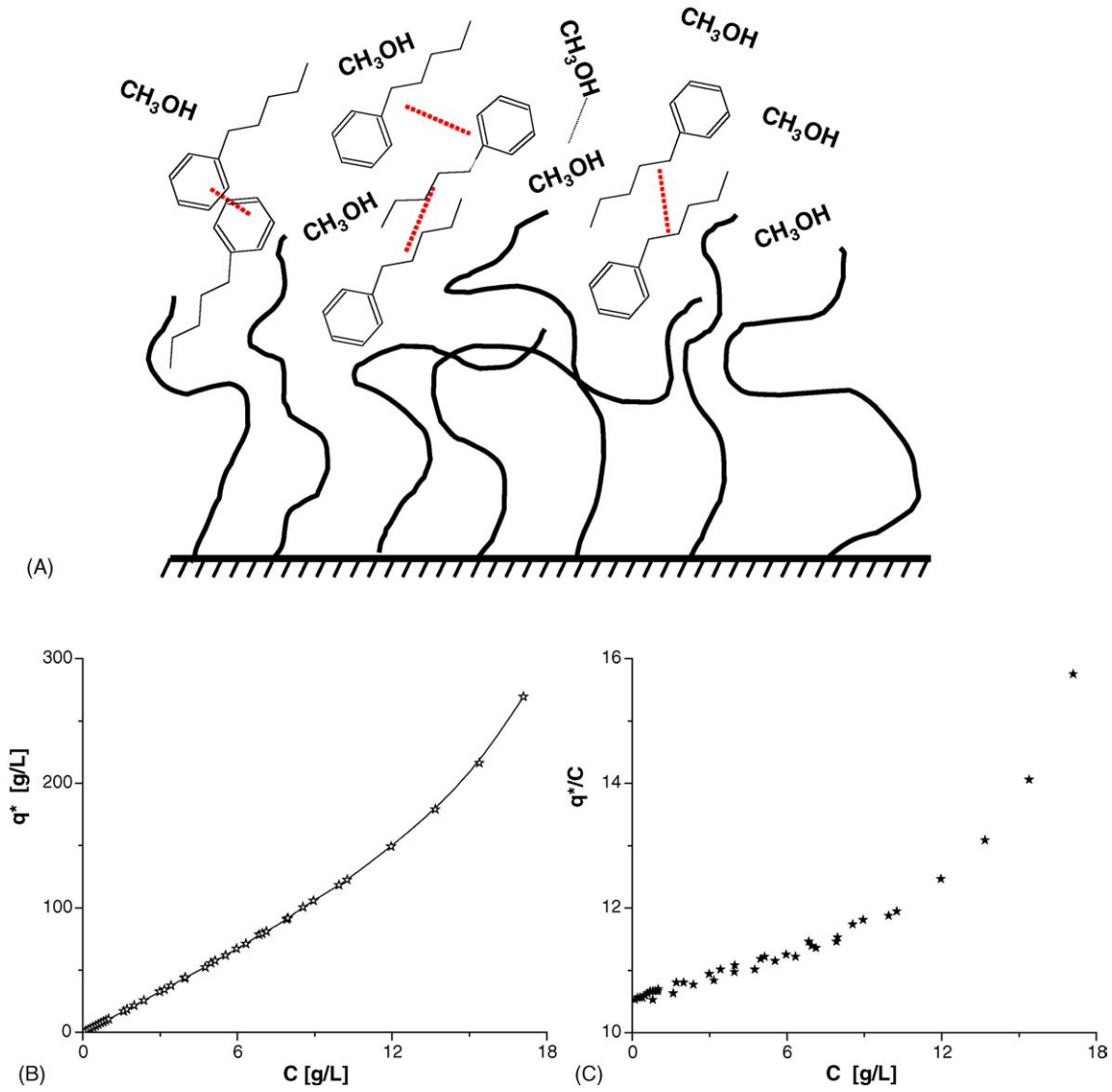


Fig. 9. Same as in Fig. 7, except for analytes whose isotherm is described by an anti-Langmuirian isotherm. Adsorbate–adsorbate interactions occur at a zero concentrations.

homogeneous surfaces and to nonideal adsorption on heterogeneous surfaces. Given the fact that we know all RPLC adsorbent surfaces to be heterogeneous, which is confirmed by the multi-modal character of the AEDs observed on all the adsorbent surfaces studied here, we need to consider only the two groups of isotherms dealing with heterogeneous surfaces. However, for the sake of simplicity, we consider a slightly different definition of the two groups. The first group accounts for the behavior of adsorption systems in which the adsorbed phase consists in a monolayer of adsorbate molecules. This does not exclude the possibility of some lateral interactions between the adsorbates in the monolayer. The second group concerns cases in which the adsorbed phase consists in a multilayer system, the solute adsorbing on the surface of the solid adsorbent and forming successive layers of the adsorbate. The advantage of this classification is that only one general isotherm equation is required for each group. As we show later, the adsorption data of all the low molecular-mass compounds that we have studied in RPLC (neutral or ionizable compounds, in the presence or absence of buffers or of supporting salts in the mobile phase) can be modeled by either one of these two groups of isotherms.

The adsorption isotherm models described below relate the analyte concentration in the bulk mobile phase to the apparent solid phase concentration. They assume that the solution contains only two components, the solute and the solvent, so they are really single-component isotherms. However, in almost every experimental case, the mobile phase is made of an organic solvent (usually methanol or acetonitrile) dissolved in water. This makes the adsorption system ternary. In principle, binary isotherms should be used, accounting for the competition between the solute and the organic modifier for adsorption. It has been shown that the simplification of replacing the binary isotherm by a single-component isotherm the coefficients

of which depend on the concentration of the modifier is legitimate in this case [67]. This is because the adsorption of the organic modifier is weak compared to that of the analyte and the competition for adsorption between the analyte and methanol is negligible. The variation of the concentration of the organic modifier essentially affects the Gibbs free energy of the analyte in the bulk mobile phase. This simplification is valid when the Henry constant of the modifier is at least five times smaller than that of the solute. The situation becomes more complex with acetonitrile and certain other modifiers the molecules of which tend to aggregate at the interface with the bonded layer, causing the formation of micro-environments in which the mobile phase properties are different from those in the bulk [113].

3.2.1. Isotherms of group I: monolayer adsorption

The general equation of the adsorption isotherms describing the formation of a monolayer of adsorbate molecules on the surface of an adsorbent takes into account (1) the heterogeneity of the surface, and (2) the possibility of adsorbate–adsorbate interactions. The degree of heterogeneity of an adsorbent is characterized by its adsorption energy distribution which quantifies the variations of the adsorption constant of the solute according to the position of the site occupied on the surface. Actual RPLC adsorbents are heterogeneous not only because the silica surface is heterogeneous but because the bonding process of the alkyl chains leads to regions of low and high density of C_{18} bonded chains, hence of high and low density of more or less nefarious, underivatized silanol groups.

The general isotherm equation for this group of models is:

$$q^* = \sum_{i=0}^{i=N} q_{S,i} \frac{b_i C + 2 I_i b_i^2 C^2}{1 + b_i C + I_i b_i^2 C^2} \tag{23}$$

where b_i is the equilibrium constant of the solute between the liquid and the solid phases, $q_{S,i}$ the saturation capacity and I_i is the adsorbate–adsorbate interaction parameter on the sites i. N is the number of homogeneous patches of the surface, characterized by distinct adsorption energies and adsorption constants. It is an index of the degree of heterogeneity of the adsorbent. Each single term in Eq. (23) corresponds to the adsorption isotherm on one of the sites that can be identified from the AED. These terms have the mathematical form of the Moreau [48] or the quadratic (the second-order Ruthven isotherm) [53] isotherm models. This equation was derived by Langmuir in 1918 [68].

Homogeneous surfaces are described by $N = 1$. The absence of adsorbate–adsorbate interactions in the ith monolayer imposes $I_i = 0$, in which case the adsorption isotherm on sites i is accounted for by a mere Langmuir isotherm model. So, Eq. (23) is a generalization of the Langmuir model of adsorption on ideal, homogeneous surfaces to the case of non-ideal adsorption on heterogeneous surfaces when the non-ideal behavior of the adsorption is moderate and the heterogeneous surface can be considered as a quilt of homogeneous surfaces.

3.2.2. Isotherms of group II: multilayer adsorption

If the experimental conditions are such that the solute can adsorb on the top of a layer of previously adsorbate molecules, an alternative model should be considered. The liquid–solid extension of the BET model, widely used in the study of gas–solid equilibria, describes well the adsorption of solute molecules from the solution onto either the bare solid surface of the absorbent or a layer of solute molecules already adsorbed. It obviously accounts for the equilibrium between these different layers. This model, however, assumes that the bare surface of the adsorbent is homogeneous and that there are no lateral interactions inside each of the monolayers. The equation of the liquid–solid BET isotherm is derived from kinetic adsorption–desorption relationships [63], with a first order kinetics. The equation obtained is:

$$q^* = q_S \frac{b_S C}{(1 - b_L C)(1 - b_L C + b_S C)} \tag{24}$$

where q_S is the monolayer saturation capacity, b_S and b_L are the equilibrium constants of the solute from the solution onto the bare surface of the adsorbent (b_S) and into a layer of adsorbate molecules (b_L). First, we note that if there are no adsorbate–adsorbate interactions between two successive layers of adsorbate molecules, Eq. (24) reduces to the Langmuir isotherm, a model which belongs to the group I. Generally, isotherms accounted for by Eq. (24) are convex upward at low concentrations, then convex downward at high concentrations (type II of isotherms according to the van der Waals classification). The reversal of the isotherm curvature, from convex upward to convex downward, takes place for a threshold concentration which corresponds to the inflection point of the isotherm. When b_L increases, and the adsorbate–adsorbate interactions increase, the position of the inflection point shifts toward lower concentrations, until it reaches $C = 0$ and the isotherm becomes strictly antilangmuirian (type III). The transition between types II and III of isotherms takes place when the second derivative of the isotherm becomes equal to 0 for $C = 0$.

$$\frac{\delta^2 q}{\delta C^2} \geq 0 \Longleftrightarrow b_L \geq \frac{b_S}{2} \tag{25}$$

4. Experimental

4.1. Chemicals

The mobile phases used in our earlier works in RPLC and mentioned in this review are all mixtures of methanol or acetonitrile with water. All solvents were of HPLC grade, purchased from Fisher Scientific (Fair Lawn, NJ, USA). The mobile phases were systematically filtered before use on a surfactant-free cellulose acetate filter membrane, 0.2 μm pore size (Suwannee, GA, USA). The hold-up tracer used was thiourea, which gives fair estimates of the total porosity of the adsorbent. All the compounds studied here were purchased from Aldrich (Milwaukee, WI, USA).

Table 2
Physico-chemical properties of the nine C_{18} bonded columns used in this work

	Column dimension (mm × mm)	Particle size (μm)	Mesopore size (Å)	Specific surface (m^2/g)	Bonding process	Carbon content (%)	Surface coverage (μmol/m^2)	End-capping
Hypurity elite	250 × 4.6	5	190	200	Monomeric	13.0	3.10	Yes
Kromasil	250 × 4.6	6	110	314	Monomeric	20.0	3.59	Yes
Luna	150 × 4.6	5	100	420	Monomeric	18.2	3.33	Yes
Symmetry	150 × 3.9	5	90	340	Monomeric	19.5	3.18	Yes
Chromolioth	100 × 4.6	Monolith 2 μm macropore	130	300	Monomeric	19.5	3.60	Yes
Discovery	150 × 4.0	5	180	200	Monomeric	12.0	3.00	Yes
Vydac	250 × 4.6	5	280	70	Polymeric	7.7	5.0	Yes
XTerra	150 × 3.9	5	86	176	Monomeric	15.2	2.50	Yes
Resolve	150 × 3.9	5	90	200	Monomeric	10.2	2.45	No

4.2. Chromatographic columns

The columns mentioned in this work are from several manufacturers and represent a large section of the market of the modern RPLC packing material. Nine brands of manufacturer-packed columns and one commercial monolithic column were used to acquire the data reported and discussed in this study. All these stationary phases were C_{18}-bonded, endcapped, porous silica. One was a "polymeric" packed column, for which a trichloro-octadecyl silane was used in the bonding process instead of a monochloro-silane. The adsorents used as stationary phases were Chromolith (Merck, Darmstedt, Germany), Discovery (Supelco, Bellefontaine, PA, USA), HyPurity Elite (Hypersil, Runcorn, UK), Kromasil (Akzo Nobel, Bohus, Sweden), Luna (Phenomenex, Torrance, CA, USA), Resolve, Symmetry and XTerra (Waters, Milford, MA, USA), and Vydac 218TP (Vydac, Hesperia, CA, USA). The main characteristics of the bare porous silica and of the materials used for the production of these brands of columns are summarized in Table 2.

4.3. Apparatus

The breakthrough curves and the overloaded band profiles of all the compounds studied were acquired using a Hewlett-Packard (Palo Alto, CA, USA) HP 1090 liquid chromatograph. This instrument includes a multi-solvent delivery system (three tanks, volume 1 L each), an auto-sampler with a 250 μL sample loop, a column thermostat, a diode-array UV-detector, and a data station. Compressed nitrogen and helium bottles (National Welders, Charlotte, NC, USA) are connected to the instrument to allow the continuous operations of the pump, the auto-sampler, and the solvent sparging. The extra-column volumes are 0.068 and 0.90 mL, as measured from the auto-sampler and from the pump system, respectively, to the column inlet. All the retention data were corrected for these contributions. The flow-rate accuracy was periodically controlled by pumping the pure mobile phase at 22 °C and 1 mL/min during 50 min, from each pump head, successively, into a volumetric glass of 50 mL. The relative error was less than 0.4%, so that we can estimate the long-term accuracy of the flow-rate at 4 μL/min at flow rates around 1 mL/min. All measurements were carried out at a constant temperature of 22 °C, fixed by the laboratory air-conditioner. The daily variation of the ambient temperature never exceeded ±1 °C.

5. Reproducibility of adsorption isotherm data

5.1. Packed columns

In this section we study the reproducibility of the adsorption isotherm data measured on different brands of packed C_{18} bonded stationary phases (seven brands), on different batches of the same brand, and on different columns from the same batch. The comparison of the adsorption data between different brands, batches, and columns from the same batch of C_{18}-bonded columns has not yet been investigated or discussed in the literature. Although there is much information there regarding the retention factors of a huge number of compounds at infinite dilution, this information is extremely limited to characterize retention mechanisms, there is little information on the isotherm data and almost none acquired systematically. So, it is most useful to compare the nonlinear adsorption properties of some of the numerous packing materials that are the fruits of uniquely researched, developed, and patented processes. This is done here. Information on the saturation capacity, the equilibrium constants, and the AEDs of these adsorbents will be presented and the differences between the retention mechanisms observed will be discussed.

5.1.1. Brand-to-brand differences

The adsorption behavior of phenol and caffeine from an aqueous solution of methanol (30:70, v/v) was measured on numerous columns from seven brands of RP-C_{18} bonded HPLC, endcapped columns. These brands are Hypurity Elite (Hypersil Inc.), Kromasil (Eka, Akzo Nobel), Luna (Phenomenex), Symmetry (Waters), Chromolith (Merck), Vydac 218TP (Grace Vydac) [69] and Discovery (Supelco) [70,71]. Figs. 10 and 11 summarize the experimental adsorption isotherm data measured. Almost all the sets of data fitted very well to the bi-Langmuir model, the exception being Discovery for which the adsorption data fitted better to a tri-Langmuir isotherm model. The choice of the best isotherm model was confirmed by the results of the calculations of the AEDs. The best isotherm parameters are reported in Tables 3 and 4 for phenol and caffeine, respectively.

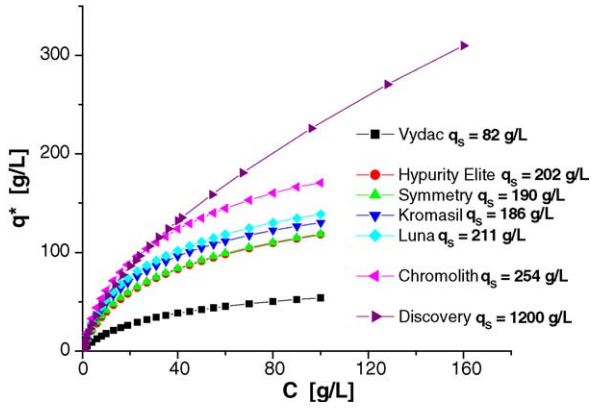


Fig. 10. Brand-to-brand reproducibility of the adsorption isotherm of phenol on seven different C_{18}-bonded stationary phases. Methanol:water, 30/70 (v/v), $T = 295$ K. Note the significant differences between these columns (saturation capacities).

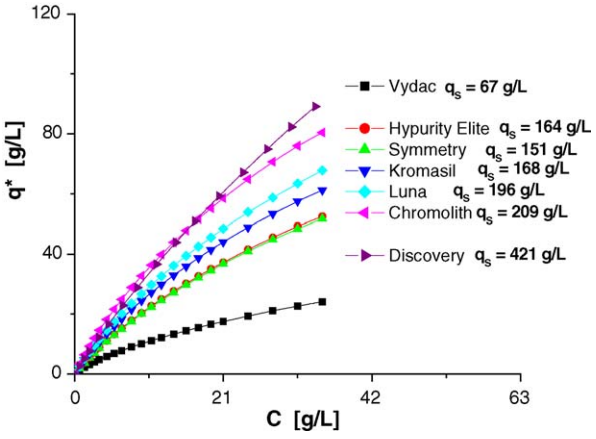


Fig. 11. Same as in Fig. 10, except for caffeine.

These results suggest that these RPLC columns have heterogeneous surfaces that contain sites of two different types (three types for Discovery), a result in agreement with the AED data discussed later.

The significant difference in the saturation capacities of the different brands is striking for both compounds. The polymeric packed column (Vydac) has the lowest capacity ($q_s < 100$ g/L), obviously, at least in a large part, because the adsorbent itself has the lowest specific surface area ($S_p = 70\,m^2/g$). Discovery has, by far, the largest total saturation capacity, followed by the monomeric, monolithic column (250 g/L). All the other monomeric packed columns have saturation capacities very close to each other (around 200 g/L). The adsorption data of butyl benzoate [63] had also shown that the monolithic RPLC adsorbent has a larger saturation capacity, around 20–30% larger. The high value measured for Discovery, around 1000 g/L and 500 for phenol and caffeine, respectively, is surprising and unexplained. These values are at least five and two times more than those of the other commercial brands, for phenol and caffeine, respectively. The physical properties of this column are not so much different from those of the other columns, however. In particular, Discovery and Hypurity are almost identical regarding the physico-chemical characteristics of their silica support, still their adsorption isotherms deviate considerably from those on the other materials, particularly at high concentrations.

Tables 3 and 4, Figs. 10 and 11 demonstrate that, for all the RPLC columns studied, the saturation capacity decreases (by ca. 20%) and the proportion of high energy sites to the overall capacity decreases markedly (ca. six-fold) from phenol ($M_W = 94$ g/mol) to caffeine ($M_W = 194$ g/mol). The comparison between the results obtained for caffeine and phenol leads to an important conclusion regarding the adsorption mechanism. The adsorption data are consistent with the relative retention of phenol and caffeine being caused by their exclusion from the stationary phase. It is at first surprising if not paradoxical that, despite the solubility of caffeine (<36 g/L) in the methanol:water solution (30:70, v/v) being much lower than that of phenol (≡ 160 g/L), the bulkier, more hydrophobic caffeine molecule is less retained than phenol. The isotherm data explain easily this strange phenomenon that linear chromatographic data cannot account for. The contribution of the high-energy sites ($q_{S,2}b_2$) to the overall retention of phenol and caffeine represents about 75 and 35% of the total retention, respectively. Even though

Table 3
Best isotherm parameters accounted for by the adsorption of phenol on seven different brands of RP-C_{18} bonded HPLC columns from a mixture of methanol and water (30/70, v/v)

Column	Vydac	Hypurity	Symmetry	Kromasil	Luna	Chromolith	Discovery
Total[a] porosity	0.66	0.71	0.59	0.61	0.63	0.84	0.72
$q_{s,1}$ (mol/L)	0.58 (0.53)	1.44 (1.52)	1.46 (1.41)	1.36 (1.35)	1.37 (1.34)	1.96 (1.82)	11.88 (13.84)
b_1 (L/mol)	1.11 (0.84)	0.71 (0.47)	0.98 (0.73)	1.37 (1.34)	0.94 (0.69)	1.28 (0.94)	0.15 (0.141)
$q_{s,2}$ (mol/L)	0.29 (0.36)	0.71 (0.85)	0.56 (0.65)	0.63 (0.64)	0.87 (1.00)	0.75 (0.98)	0.83 (0.88)
b_2 (L/mol)	7.31 (5.67)	7.72 (6.08)	10.9 (6.99)	11.8 (11.6)	9.11 (7.36)	11.8 (7.85)	6.69 (6.81)
$q_{s,3}$ (mol/L)	–	–	0.48[b] (n.d.)	–	–	0.30[c] (0.30)	0.06 (0.03)
b_3 (L/mol)	–	–	18.8[b] (n.d.)	–	–	37.8[c] (46.7)	28.7 (45.1)
$\frac{q_{S,2}}{q_{S,1}+q_{S,2}}$	33% (40%)	33% (36%)	28% (32%)	32% (32%)	39% (43%)	28% (35%)	6.5% (6.5%)
$\frac{b_2}{b_1}$	7 (7)	11 (13)	11 (10)	9 (9)	10 (11)	9 (8)	45 (48)

The values in parentheses are the isotherm parameters derived from the calculation of the AED.

[a] Derived from the injection of thiourea.
[b] Measured with 20% methanol in the mobile phase.
[c] Measured with 15% methanol in the mobile phase.

Table 4
Best isotherm parameters accounted for by the adsorption of caffeine on seven different brands of RP-C_{18} bonded HPLC columns from a mixture of methanol and water (30/70, v/v)

Column	Vydac	Hypurity	Symmetry	Kromasil	Luna	Chromolith	Discovery
Total[a] porosity	0.66	0.71	0.59	0.61	0.63	0.84	0.72
$q_{s,1}$ (mol/L)	0.33 (0.34)	0.79 (0.80)	0.74 (0.76)	0.82 (0.84)	0.95 (1.02)	1.03 (1.02)	2.15 (3.27)
b_1 (L/mol)	2.69 (2.30)	2.27 (2.26)	2.64 (2.43)	2.84 (2.74)	2.57 (2.31)	3.16 (3.2)	1.49 (1.13)
$q_{s,2}$ (mol/L)	0.02 (0.02)	0.05 (0.05)	0.03 (0.04)	0.05 (0.04)	0.06 (0.06)	0.05 (0.04)	0.02 (0.02)
b_2 (L/mol)	52.5 (30.2)	20.6 (21.2)	26.3 (22.0)	27.0 (28.0)	24.0 (22.7)	35.4 (38.2)	39.6 (36.3)
$\frac{q_{S,2}}{q_{S,1}+q_{S,2}}$	6% (7%)	6% (6%)	4% (6%)	5% (5%)	6% (6%)	5% (4%)	1% (0.7%)
$\frac{b_2}{b_1}$	20 (7)	9 (13)	10 (10)	10 (9)	9 (11)	11 (8)	45 (27)

The values in parenthesis are the isotherm parameters derived from the calculation of the AED.

[a] Derived from the injection of thiourea.

the retention contribution of the low-energy sites is larger for caffeine than for phenol, this effect does compensate for the reduction of the contribution of the sites of type 2. The major difference is that caffeine cannot access as many type 2 sites as phenol can. The larger size of the caffeine molecules causes their exclusion from narrow adsorption sites located between randomly coiled alkyl chains that may accommodate phenol molecules.

Obviously, the comparison of the adsorption data obtained with different brands of RPLC packing materials shows qualitative similarities (they have the same adsorption model, except for Discovery for which the model is still similar), as we should expect from materials that are chemically quite similar. However, there are significative differences in the values of the isotherm parameters. These differences are not fully explained by differences between the physico-chemical characteristics of the bare silica. Of critical importance for preparative purposes, the column capacity varies widely. The Discovery adsorbent shows an exceptionally high saturation capacity and may warrant attention from those interested in sample preparation. Unfortunately, the total saturation capacity is of modest importance compared to the saturation capacity of the high energy sites [70,72]. These sites fill first and, when they become saturated, retention decreases markedly causing the changes in peak profile associated with column overloading [124].

5.1.2. Batch-to-batch reproducibility

Isotherm data were acquired with columns of six Kromasil-C_{18} batches (batches E6019, E6103, E6104, E6105, E6106, and E6436). These batches are the same as those studied earlier, under linear conditions, by Kele [73]. The C_{18} derivatization process and the endcapping process were carried out separately on different batches of bare silica. The physico-chemical properties of the bare silica and the corresponding C_{18} derivatized silica are given in Table 5. The accurate adsorption data were measured by FA only on column E6019, for seven different chromatographic systems (different mobile phase compositions and compounds) exhibiting several different isotherm shapes [74]. The best isotherm models were derived according to the method described earlier (see Section 2). A different method was used to measure the adsorption isotherm with the columns of the five other batches, for the sake of saving on the time and the chemicals needed to obtain the data. The inverse method [37,75] was used. Overloaded band profiles were recorded and used to estimate the best parameters of the isotherm model that fitted the data of the same system on column E6019. This method uses the equilibrium-dispersive model of chromatography to calculate chromatographic band profiles and optimizes the parameters of the isotherm model to achieve the best possible agreement between experimental and calculated band profiles. The method relies on the assumption that the materials packing the different columns are too similar for the isotherm model to change from one column to the others. The differences between two columns will result only in numerical differences between the isotherm parameters and, in the cases of columns from different production batches, these differences should be small.

Table 5
Physico-chemical properties of the 10 packed Kromasil-C_{18} columns (Eka)

Columns	Bare silica batch					Silica-C_{18} batch	
	Particle size (μm)	Particle size distribution (90:10, % ratio)	pore size (Å)	Surface area (m^2/g)	Na, Al, Fe content (ppm)	Total carbon (mass %)	Surface coverage (mmol/m^2)
E6019 (I)	5.98	1.44	112	314	11; <10; <10	20.00	3.59
E6021 (II)	5.98	1.44	112	314	11; <10; <10	20.00	3.59
E6022 (III)	5.98	1.44	112	314	11; <10; <10	20.00	3.59
E6023 (IV)	5.98	1.44	112	314	11; <10; <10	20.00	3.59
E6024 (V)	5.98	1.44	112	314	11; <10; <10	20.00	3.59
E6103 (VI)	5.98	1.44	112	314	11; <10; <10	19.65	3.51
E6104 (VII)	5.98	1.44	112	314	11; <10; <10	19.85	3.55
E6105 (VIII)	6.03	1.38	112	322	15; <10; <10	20.00	3.50
E6106 (IX)	6.24	1.48	107	333	23; <10; <10	20.60	3.52
E6436 (X)	6.11	1.46	114	313	15; 13; 14	19.80	3.55

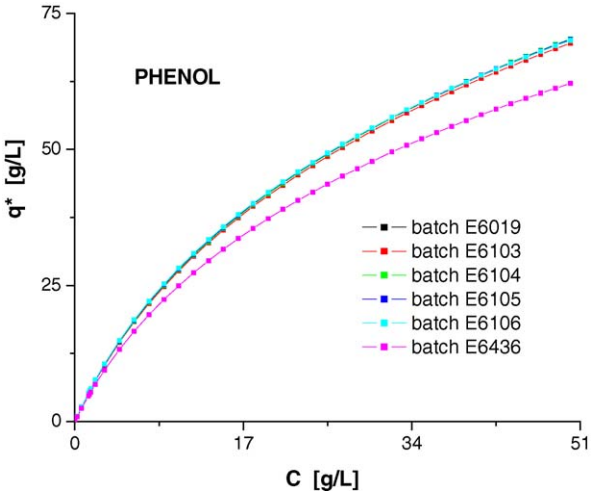


Fig. 12. Batch-to-batch reproducibility of the adsorption isotherm of phenol (langmuirian isotherm) measured on six different batches of Kromasil-C_{18} bonded phases. Methanol:water, 45/55 (v/v), $T = 295$ K. Except for the batch E6436, the level of reproducibility is very good.

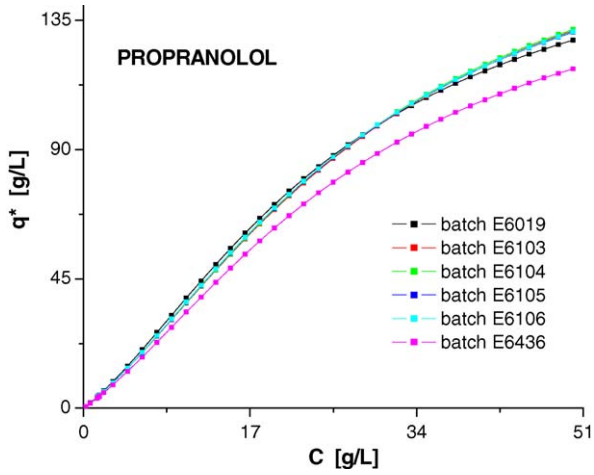


Fig. 13. Same as in Fig. 12, except for the compound propranolol (S-shaped isotherm) and the mobile phase composition (40% methanol, v/v).

We report here only the data characterizing the reproducibility of the adsorption isotherms of three compounds, one with a convex upward isotherm (phenol, bi-Langmuir isotherm), one with an S-shaped isotherm (propranolol, Moreau isotherm), and one with a convex downward isotherm (ethyl benzene, BET isotherm). Figs. 12–14 show the isotherms of these three compounds calculated with the optimized sets of isotherm parameters for each of the six batches and the three compounds. The reproducibility of the adsorption isotherm is excellent for five out of six batches. The batch E6436 exhibits obvious but still modest differences, with a lower saturation capacity than average for the five other columns (138 g/L versus 156 g/L for phenol, 170 g/L versus 186 g/L for propranolol, and 156 g/L versus 176 g/L for ethyl benzene. There are no clear physical explanations for this observation that would be consistent with the physico-chemical parameters of the six columns (see Table 5). According to Felinger et al. [75], the reproducibilities of both the saturation capacities q_S and the equilibrium constants b of these isotherms is between 1.2 and 3% when the data for column E6436, considered as an outlier, are omitted. This degree of reproducibility is remarkable given the complexity of both the nonlinear phenomenon accounting for the propagation of high concentration bands and the manufacturing process of porous silica and its bonding. A similar degree of reproducibility was observed for the isotherm models and parameters of aniline (Jovanović isotherm), caffeine (bi- Langmuir isotherm), and theophylline (Tóth isotherm) [75]. We can expect comparable batch-to-batch reproducibility levels for the other manufactured brands of RPLC columns.

5.1.3. Column-to-column reproducibility

The reproducibility of the adsorption isotherms of phenol, propranolol, and ethyl benzene on five columns packed with the same batch of Kromasil-C_{18} packing material were compared. The isotherm data were measured using the same inverse method as that used for the study of the batch-to-batch reproducibility discussed above. The differences observed are explained by the limitations of the reproducibility of the packing process (fluctuations of the exact column dimensions and of the packing density [74,75]), not by the limited reproducibility of the bonded silica. The best isotherms calculated from the isotherm parameters given by the inverse methods are shown in Figs. 15–17 for phenol, propranolol, and ethylbenzene respectively. In this case, there were no outliers like in the previous one. The column-to-column reproducibility, however, is not perfect. The five columns packed with the same material have definitely slightly different total porosities [74] after their packing with the slurry method. The retention factors at infinite dilution are also slightly different, suggesting slightly different phase ratios. The standard deviations of the best isotherm parameters for the column-to-column reproducibility were the same as those found for the batch-to batch reproducibility [75]. This confirms the high level of reproducibility of RPLC adsorbents (at least for the C_{18}-bonded Kromasil column). The main difference between columns originates in small fluctuations of the packing density and of the tubing size that take place during the packing

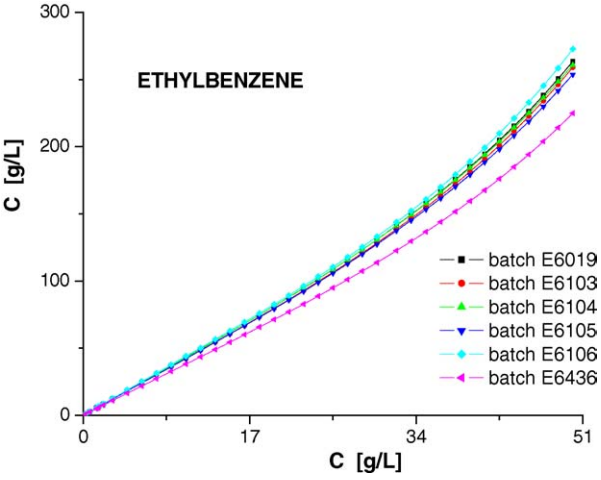


Fig. 14. Same as in Fig. 12, except for the compound ethylbenzene (anti-langmuirian isotherm) and the mobile phase composition (80% methanol, v/v).

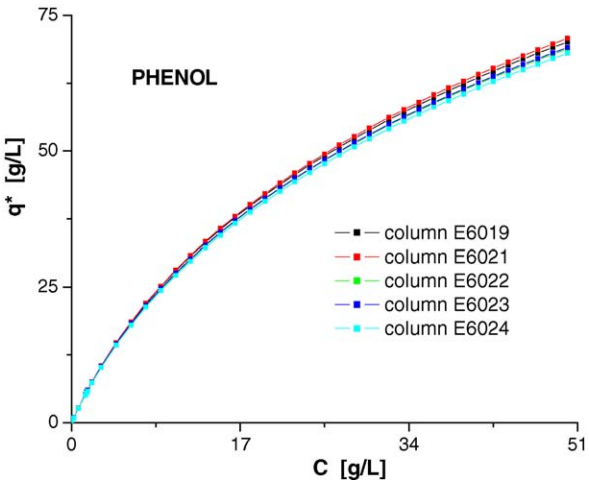


Fig. 15. Column-to-column reproducibility of the adsorption isotherm of phenol (langmuirian isotherm) measured on five different columns packed with the same batch of Kromasil-C_{18} bonded material. Note the very good reproducibility level.

process, as suggested by the small column-to-column variations of the hold-up times.

5.2. Monolithic columns

The reproducibility of the adsorption data obtained with a lot of six new monolithic columns (Chromolith-C_{18}, Merck, Germany) was also measured. Monolithic materials have gained a considerable interest in the last 5 years and they are now widely accepted [76]. Their application for preparative HPLC applications is under intense study. The same method of column characterization as that used for packed columns was applied [77]. With monolithic columns, it is not possible, however, to study separately column-to-column and batch-to-batch reproducibilities because of the characteristics of the manufacturing process (there is only one column per batch). The results obtained demonstrated that four of the six columns gave nearly identical numerical values for the isotherm parameters, independently of the nature of the compound studied and of the corresponding adsorption isotherm (convex upward, S-shaped, or convex downward). For these four columns, the reproducibility was better than 5 and 2.5% for the low and the high concentration overloaded band profiles. Two other columns gave different results, with retention times between 6 and 15% larger and 2 and 7% lower, respectively, than those obtained with the four similar columns. The differences appeared to be correlated to differences in the column total porosities ϵ_t of these six columns. There is an average maximum absolute difference of 0.025 in ϵ_t or a difference of about 35 μL in the column hold-up volume. This may be small but it is sufficient markedly to affect the reproducibility of the retention properties of these columns. At this stage, it is likely that the preparation of monolithic columns, which are commercially available for only a mere 5 years, is still somewhat less reproducible than that of the manufacturing of packing materials [74]. Systematic measurements carried out with a larger number of monolithic columns and more compounds would be required to achieve a more precise insight on the level of reproducibility of these new chromatographic columns.

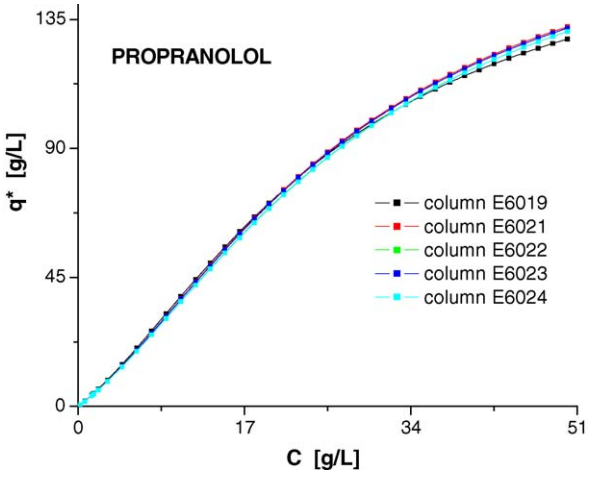


Fig. 16. Same as in Fig. 15, except for the compound propranolol (S-shaped isotherm) and the mobile phase composition (40% methanol, v/v).

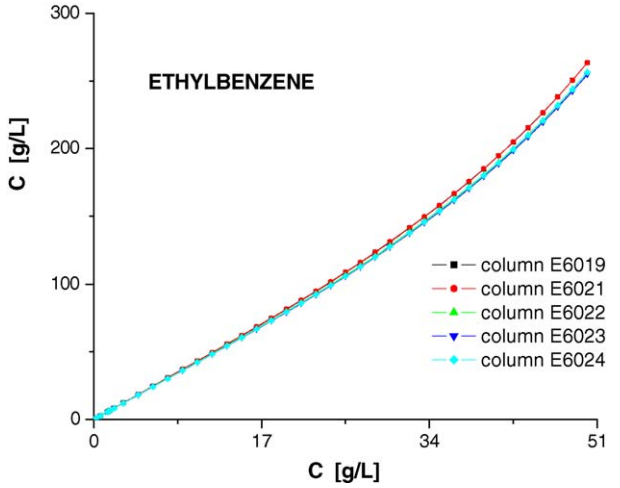


Fig. 17. Same as in Fig. 15, except for the compound ethylbenzene (anti-langmuirian isotherm) and the mobile phase composition (40% methanol, v/v).

6. Heterogeneity of RP-C_{18} HPLC columns

Although all chromatographers realize that the surfaces of packing materials are somewhat heterogeneous and that this defect may explain numerous difficulties, the field has been graced by far more spirited discussions that sound data. The problem of the heterogeneity of modern RPLC columns has intrigued and aroused but has yet brought few sound, quantitative answers. There are no doubts that RPLC materials exhibit heterogeneous surfaces, that the silica surface has embedded inorganic impurities (e.g., aluminum, iron, boron, sodium), that it is rough down to the molecular level, and that it is sprinkled with a finite density of nefarious silanol groups. However, if attention has focused on these groups, little or no interest has been devoted to the structure of the bonded alkyl layer in connection with this surface heterogeneity. Most cogent conclusions originate from the analysis of chromatographic data that were obtained at infinite dilution,

hence under linear conditions. Tests based on linear elution of weak or strong basic compounds reveal high-energy sites of interaction between these bases and the acidic silanol groups, leading to an excessively high retention (thermodynamic contribution) and/or to strong peak tailing (thermodynamic and/or kinetic contributions). The slow kinetics of the desorption process from these high-energy silanol sites has been the most widespread physical interpretation of peak tailing in chromatography. It is only recently that chromatographers begin to understand how pervasive and obnoxious is the contribution of surface heterogeneity to elution profiles at low concentrations [70,72]. Yet, this contribution is of thermodynamic origin.

Recent investigations based on the measurement of adsorption isotherms and on the appropriate treatment of these data by calculating the AED of the surface (see Section 2.2) have brought up new insights and opened new lines of thinking regarding the interpretation of peak tailing in chromatography [45,78]. Most importantly, it was demonstrated that it is extremely rare in RPLC that a common, simple isotherm model (e.g., the Langmuir or Jovanovic isotherm models) can ever be applied to account for actual adsorption data (note that this statement is not true for other modes of chromatography, e.g., normal phase, nor for big molecules, e.g., proteins in RPLC).

Adsorption isotherm data of selected probes may provide a quantitative measure of the heterogeneity of the surface of adsorbents. These data can be fitted to isotherm models and can be used to calculate the AED of the surface for these probes. The determination of the fractions of the adsorbent surface onto which the compound is adsorbed with a given adsorption energy informs on which sites are preponderant. The difference between the adsorption energies on different types of sites informs on the nature of the different interactions between the surface and the probe. We review now the results on the heterogeneity of common stationary phases based on the adsorption data for two neutral compounds, phenol and caffeine. These two compounds have different molecular weights (94 and 194 g/mol) and different sizes, the former containing one, the second two aromatic rings. Because of their relatively high polarity (see Section 3), their adsorption isotherms on C_{18}-bonded silicas are always convex upward. Accordingly, the overall isotherm can be decomposed into the sum of several elementary Langmuir isotherms. Each single langmuirian contribution describes a distinct region of the solid surface. The higher the number of Langmuir terms, the higher the degree of heterogeneity of the bonded material.

6.1. The necessity of calculating the AED

It is not unusual that the same adsorption isotherm data acquired by FA fit both a unimodal and a multi-modal adsorption isotherm model, both with reasonably large values of the Fisher coefficient. This leads to model indetermination. Yet, there is but one isotherm model that is physically correct. It may be different from the models tried, different from the models that we know, and we may never find it. We must try to approach it and find a model that differs from the true one in ways lesser than the errors of measurements. Such a case was encountered, for example, in the study of the adsorption of phenol and caffeine on Kromasil-C_{18} from mixtures of methanol and water (45/55 and 30/70, v/v, respectively) [79]. The adsorption of both phenol and caffeine can be well described using either a unimodal isotherm model, the Tóth model [80,81], or a bimodal one, the bi-Langmuir model [82]. The former isotherm has one rather broad and unsymmetrical energy mode in its AED while the second one has two very narrow modes that are well resolved. This implies two quite different models of heterogeneity of the adsorbent surface that are not compatible. An independent approach is necessary to choose between them, for example another method of treatment of the adsorption data.

The calculation of the AED brings this new piece of information. The AEDs obtained for caffeine and phenol are both bimodal, which suggests to ignore the unimodal Tóth isotherm. Conclusion on the heterogeneity of the surface is then straightforward. Both phenol and caffeine may adsorb on two distinct types of sites that have quite different adsorption energies, each of them nearly homogeneous. They do not adsorb on sites of a single type having a continuous, somewhat broad energy distribution, tailing toward the low energies. Obviously, this conclusion has important implications regarding the adsorption mechanism of these two compounds and, more generally, regarding retention mechanisms in RPLC. For example, it is noteworthy that the difference between the adsorption energies on the two types of sites is only about 5 kJ/mol. Although this difference is important and will explain the poor overloading behavior of the stationary phase under slightly overloaded conditions [70,72], it is small as far as molecular interactions are concerned. It is likely that the physico-chemical nature of the interactions is the same on both types of sites, 1 and 2. The difference is too small to be consistent with one type of sites corresponding to van der Waals interactions (bonded alkyl chains and molecules of phenol or caffeine) and the other to strong ion-exchange interactions or to interactions with an isolated silanol group. Furthermore, any hydrogen-bonding interactions with an isolated silanol are very unlikely because of the relative importance of the area covered with type 2 sites. According to the AED calculations, the fractional surface areas of these sites represent 23 and 2.6% of the total available accessible surface area of the adsorbent, for phenol and caffeine, respectively. Silanol groups probably cover close to 23% of the adsorbent surface area after completion of the C_{18} derivatization and of the endcapping of the silica surface. Using the results of NMR measurements, Scholten et al. [83] showed that after the derivatization of the silica surface with dimethyl-octadecylsilane, 21% of the surface groups were shielded silanols, 20% were free silanols, and 59% were alkylsilane ligands. The endcapping process probably eliminates a large fraction of the free silanols but leaves unaffected the shielded ones so that, after derivatization and endcapping, about 35% of silica surfaces is covered with shielded (hence unaccessible for interactions with solutes) silanol groups, 5% being free silanols, and 60% of these surfaces is covered with octadecyl ligands. Accordingly, the accessible silanol groups cannot account for 23% of the adsorbent area after completion of the C_{18} derivatization and of the endcapping of the silica surface. Finally, it appears that type 2 sites are certainly size selective. The larger caffeine molecules can visit only one tenth of the sites to which

the smaller phenol molecules have access. Size selectivity may explain the difference in the relative abundance of the high-energy sites for phenol and for caffeine.

Finally, it is important to observe that an AED is a characteristic of both the surface studies and the probe. The features of an AED do not directly mirror the surface heterogeneity of the material. They depend on which aspects of the surface are observed or probed. First, isotherm measurements and AED calculations remain insensitive to any surface heterogeneity the size of which is small compared to the dimensions of the molecules of the probe solute because the interactions between the surface and the probe molecule average out the effects of these small, local heterogeneities. A heterogeneity of the electrostatic field above the adsorbent surface is observed only if it affects the integral of all the interactions of the surface with the probe molecule. The AED of an alkane will have far fewer distinctive features than that of an amino acid (assuming that both can be acquired). The true surface heterogeneity cannot be obtained by chromatographic measurements, only the heterogeneity of the interactions of several probe molecules with this surface can be. The AED gives only a precise idea of the way in which the analyte interacts with the surface, depending on the nature of the solute and on the mobile phase composition. The degree of heterogeneity of RPLC adsorbents can be compared only when using the same probe molecule and mobile phases of similar composition. The knowledge acquired is limited to the main interactions involved in the retention of the compound studied. In the next section, we compare the results obtained with eight different brands of RPLC materials using phenol and caffeine and the same solution of methanol and water.

6.2. Comparison of the surface heterogeneity of various types of C_{18}-bonded columns

The AED of a probe on an adsorbent informs on whether the adsorption model that accounts for the adsorption data has a unimodal or a multi-modal energy distribution and on the relative homogeneity of the different adsorption sites located on the surface studied. In the case studied here, the first results showed that the adsorption of low molecular weight compounds on C_{18}-bonded endcapped silica takes place on two distinct types of sites. This conclusion is valid for eight of the nine columns studied. This result should be checked on numerous other commercial columns in order to find out what is its degree of generality and whether there are exceptions that would inform better on the nature of the different types of adsorption sites that can be identified on these adsorbents.

We first discuss studies on the impact of the endcapping process after derivatization of the silica and on the morphology of the solid support (monolithic or packed particles) on the surface heterogeneity of RPLC adsorbents. Then we compare the heterogeneity of various brands of modern endcapped C_{18}-bonded packing materials.

6.2.1. Endcapped and non-endcapped materials

The adsorption isotherms of phenol and caffeine were measured by FA and their AED calculated on the non-endcapped Resolve-C_{18} and the endcapped Symmetry-C_{18} and XTerra-C_{18}, all three manufactured by the same company, Waters (Milford, MA, USA). The same mixture of methanol and water (25/75, v/v) was used as the mobile phase. The abundance of isolated silanols increases from XTerra-C_{18} (almost nonexistent, according to reference [84]) to Symmetry-C_{18}, and to Resolve-C_{18}. However, the adsorption behavior of phenol is almost the same on these three different adsorbents. A bimodal energy distribution was found for all three adsorbents (Fig. 18). The best corresponding parameters are given in Table 6. Qualitatively, the degree of heterogeneity of these three adsorbents is identical. The values of b_1 and b_2 and their ratios are very close for the three materials (Table 6). We note, however, significant differences in the degree of heterogeneity of the two types of sites. Symmetry has certainly the narrowest modes for both types of sites. Their mode in the AED being slightly wider, the high-energy sites of XTerra are slightly more heterogeneous than those of Symmetry but the low-energy sites are much wider, thus more heterogeneous. The converse is true for Resolve. The saturation capacities of both sites are lower on Symmetry than on the other two materials, XTerra and Resolve, probably because of the lower total porosity of Symmetry. The presence of non endcapped regions on Resolve does not affect the retention of phenol, probably because these regions are preferentially covered by the polar molecules of water or methanol. Some isolated silanols may be trapped within the C_{18}-bonded layer but they must remain inaccessible to the analyte which does not seem to be able to form strong interactions with them. A fraction of 25–30% of the surface which can be in contact with phenol molecules is covered with high-energy sites but the difference between the adsorption energies on the two types of sites is only about 5 kJ/mol. Accordingly, it makes little difference for phenol whether the column is endcapped or not, which is consistent with phenol not being retained on pure silica [45]. This molecule is not small enough to probe the fine details of the surface heterogeneity induced by the absence of endcapping. The size of the probe is not appropriate to detect any differences between endcapped and non-endcapped adsorbents.

In contrast (see Fig. 19), caffeine shows significant differences between the two types of adsorbent. Its AEDs on XTerra and Symmetry are bimodal, like those of phenol, consistent with the bi-Langmuir model accounting well for the adsorption data of caffeine. On Resolve, however, the AED is tetramodal and a tetra-Langmuir isotherm best describes the adsorption isotherm behavior of caffeine. The relative abundance of the high-energy type 2 sites to the low-energy type 1 sites is far smaller for caffeine than for phenol on all three stationary phases. It decreases in the same time as the size of the probe which suggests its exclusion from sites that are more deeply located in the C_{18}-bonded layer. Note, however, that the relative abundance of types 2 and 1 sites is higher on the non-endcapped Resolve (15% versus 5 and 9% for Symmetry and Xterra). The endcapping certainly eliminates a significant fraction of accessible sites of type 2. The most striking result obtained is the apparition of two new high-energy sites in small and very small amounts, respectively, on Resolve. The energy difference, ΔE, between sites of types 1 and sites of types 3 and 4 is about 10 and 20 kJ/mol, respectively. The sites of

Table 6
Best isotherm parameters accounted for by the adsorption of phenol and caffeine on three different brands of RP-C_{18} bonded HPLC columns from a mixture of methanol and water (25/75, v/v)

Column	Probe solute					
	Phenol			Caffeine		
	Resolve	Symmetry	XTerra	Resolve	Symmetry	XTerra
End-capped	No	Yes	Yes	No	Yes	Yes
Total[a] porosity	0.63	0.60	0.64	0.63	0.60	0.64
$q_{s,1}$ (mol/L)	2.01 (2.01)	1.46 (1.41)	2.00 (2.00)	0.81 (0.81)	0.74 (0.76)	0.79 (0.79)
b_1 (L/mol)	0.65 (0.67)	0.98 (0.73)	0.54 (0.52)	3.36 (3.72)	2.64 (2.43)	2.89 (2.75)
$q_{s,2}$ (mol/L)	0.69 (0.67)	0.56 (0.65)	0.65 (0.68)	0.14 (0.11)	0.03 (0.04)	0.08 (0.09)
b_2 (L/mol)	9.1 (9.4)	10.9 (7.0)	12.2 (10.9)	27.9 (33.6)	26.3 (22.0)	29.9 (25.4)
$\frac{q_{S,2}}{q_{S,1}+q_{S,2}}$	26% (25%)	28% (32%)	25% (25%)	15% (12%)	4% (5%)	9% (10%)
$\frac{b_2}{b_1}$	14 (14)	11 (10)	10 (10)	8 (9)	10 (9)	10 (9)
$q_{s,3}$ (mol/L)	–	–	–	0.014 (0.011)	–	–
b_3 (L/mol)	–	–	–	237 (267)	–	–
$q_{s,4}$ (mol/L)	–	–	–	0.00030 (0.00028)	–	–
b_4 (L/mol)	–	–	–	13000 (13550)	–	–

The values in parentheses are the isotherm parameters derived from the calculation of the AED.

[a] Derived from the injection of thiourea.

type 3 do not seem to be due to strong interactions between either isolated or free silanols and caffeine [45]. These sites are probably strongly hydrophobic sites on which the analyte is deeply embedded in the layer of C_{18}-bonded chains. The nature of the adsorption sites of type 4 is subject to discussion. The high adsorption energy on those sites suggests that strong interactions take place, possibly with some isolated silanols within the hydrophobic layer. The number of sites of type 4 (0.30 mmol/L) corresponds to a surface density of about 1 nmol/m^2, a value which may be consistent with residual, isolated silanols. However, such interactions are absent with the smaller analyte phenol, which has a better access to these silanols but does not seem to interact strongly enough with them. These high energy sites contribute significantly to the retention of the compound. In the case of caffeine, the contributions to the retention of each type of site are nearly the same. Because of the very high energy and very low capacity of the sites of type 4, there is an extensive peak tailing at low concentrations, a tailing that is of thermodynamic origin and that cannot be eliminated because linear behavior is not yet achieved at the detection limit. This also explains why the elution order of caffeine and phenol is reversed on Resolve, on the one hand, and on XTerra and Symmetry, on the other.

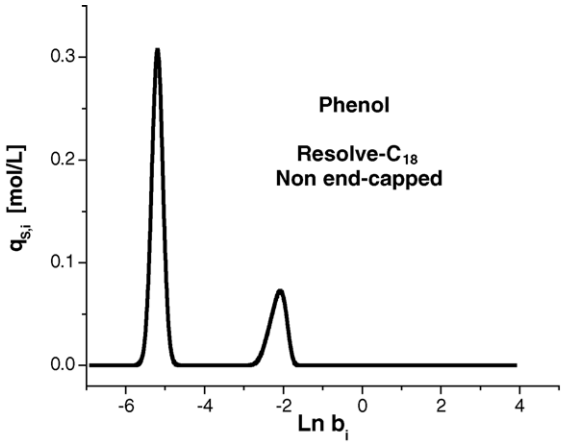
As a result, it appears that caffeine is more suitable than phenol to assess the degree of heterogeneity of the C_{18}-bonded surfaces of non-endcapped materials. The endcapping of the adsorbent certainly helps to make the surface of the adsorbent more homogeneous, not only by removing a fraction of the nefarious silanol groups, but also by smoothing out some of the roughness of the C_{18}-bonded phase structure.

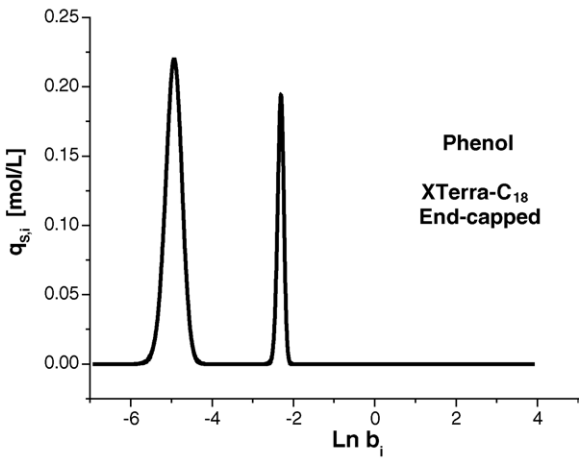
6.2.2. Monolith and packed columns

The adsorption isotherms of phenol and caffeine and their corresponding adsorption energy distribution have also been measured on the endcapped C_{18}-Chromolith adsorbents [78]. Because of the very high total porosity of the column (ca. 0.87), there is a low amount of solid material in the column and the methanol concentration had to be decreased to 15% to achieve a retention compatible with a sufficient accuracy of the FA data. It has been shown that the methanol concentration does not change the number of types of adsorption sites but essentially affects the relative proportion and the adsorption energies of these sites [85–87]. It was also shown that the relative abundance of the high energy sites of the endcapped Kromasil adsorbent increases with decreasing methanol concentration while the adsorption constant follows the linear solvation strength model (LSSM) [86]. A similar result should be expected for Chromolith. Fig. 20 shows that the AEDs of phenol and caffeine on Chromolith are trimodal and quadrimodal, respectively. The lowest two energy modes appear to be the same as those observed on classical packed columns (Figs. 18 and 19). They could correspond to the adsorption at the interface between the solution and the hydrocarbon layer and to the dissolution of the analyte inside the bonded layer, respectively. Despite the fact that these columns are endcapped and derivatized with C_{18}, much higher energy sites are observed, with adsorption constants 80 and 1500 times larger than on type 2 sites. The particular morphology of the Chromolith silica (monolithic columns are often viewed as made of interconnected porous-silica cylinders [88] while packed columns as interconnected porous spheres) may lead to regions where the density of the bonded alkyl chains is significantly different from that in other regions (e.g., on flat and curved surfaces). This structure might favor the formation of a few new hydrophobic adsorption sites. However, the formation of these new sites or of part of them may also be a consequence of the reduction made in the methanol content of the mobile phase.

6.2.3. Monomeric and polymeric columns

Monomeric phases have become by far the most popular RPLC stationary phases. They can be obtained by reaction of a monofunctional silane (e.g., an alkyldimethylchlorosilane) with silica to form a siloxane bridge between the organic and silica





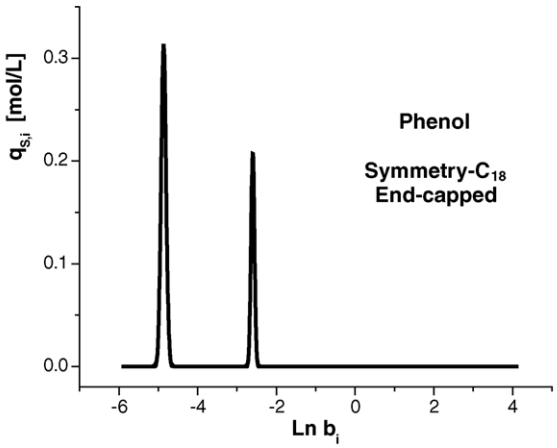
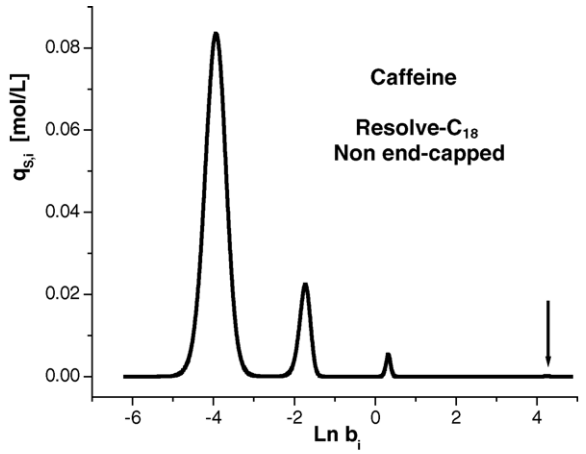
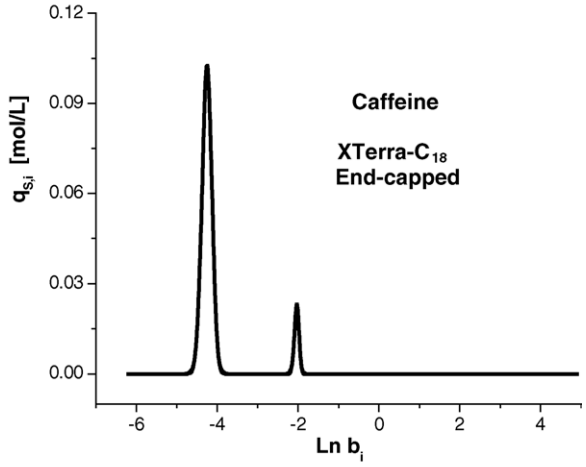


Fig. 18. AEDs of phenol on non-endcapped (Resolve-C_{18}) and endcapped (XTerra-C_{18} and Symmetry-C_{18}) stationary phases. Methanol:water, 30/70, (v/v). Note the same bimodal AED for each column. Reproduced with permission from [78] (Figs. 1A and 2A) and [69] (Fig. 4A). ©2003, American Chemical Society.





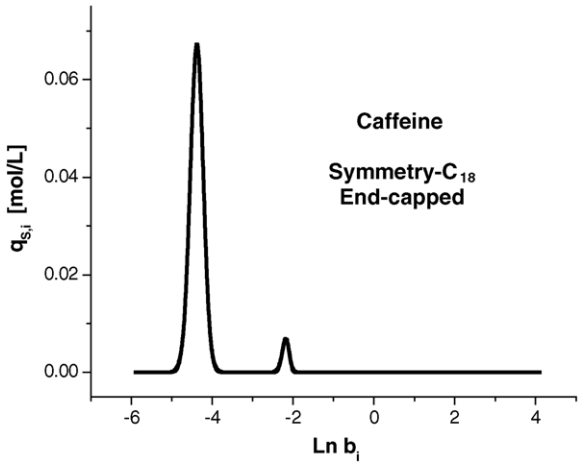


Fig. 19. Same as in Fig. 18, except for caffeine. Note this time the appearance of two new adsorption sites with the non-endcapped column. Reproduced with permission from [45](Figs. 6A and 8A) and [69] (Fig. 4B) ©2003, American Chemical Society.

moities. Depending on the degree of control of the reaction conditions, it is possible to achieve a surface coverage of between 2.0 and 4.0 μmol/m^2 (see Table 2). With these adsorbents, one surface silanol is covalently bound at most to one alkyl chain, which limits the bonding density to around 4.0 μmol/m^2. To achieve higher chain densities, in order to prevent access of the solute to the bare silica surface, to increase column stability, or to improve chromatographic selectivity, another method of bonding alkyl chains to the silica surface is possible. Polymeric phases are prepared from di- or tri-functional silanes. Their use leads to higher chain densities but also to a more complex surface chemistry. Because the silane reagent may either anchor to the surface (Si–OH) or to another silane by hydrolyzation, a polymeric network will form that extends out from the silica surface. Table 2 shows that the chain density of the polymeric

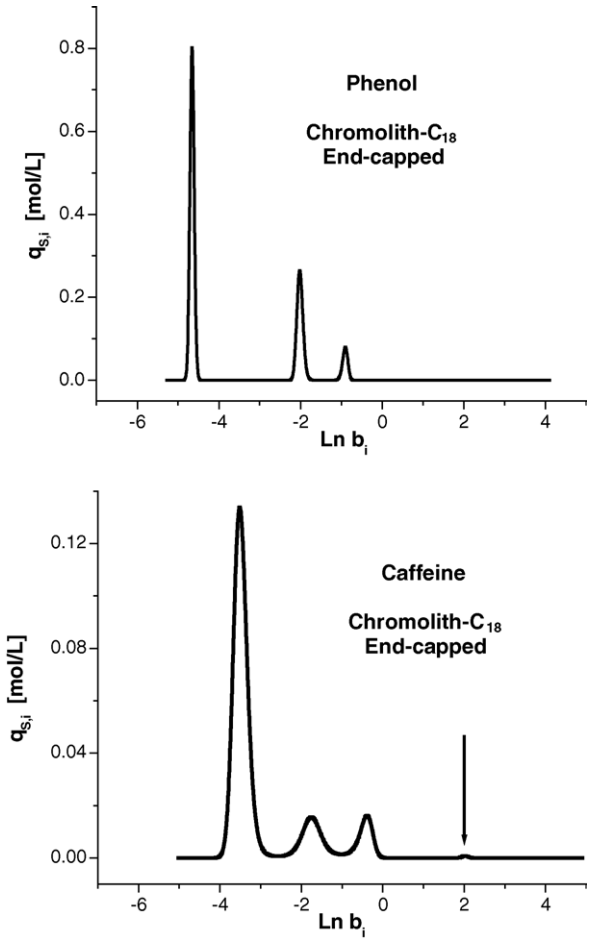


Fig. 20. AED of phenol on the Chromolith-C_{18} adsorbent. Methanol:water, 25/75 (v/v). Note the higher degree of heterogeneity of monolithic column by comparison to spherical packed columns (see Figs. 18 and 19). Reproduced with permission from [78] (Figs. 3 and 8).

phase Vydac is nearly twice greater that those of the monomeric phases (5 versus an average of about 3.0 μmol/L).

The results of the FA measurements and the isotherms obtained for phenol and caffeine (Tables 3 and 4) are quite sensitive to this difference in the structure of the hydrophobic bonded layer. The isotherms are still bi-Langmuir but the two saturation capacities, $q_{S,1}$ and $q_{S,2}$, are much lower on the polymeric phase, in part because of the lower specific surface area of the bare silica (70 m^2/g versus an average of 280 m^2/g for the monomeric phases). Note, however, that the four-fold reduction in specific surface area does not correlate well with the two-fold reduction in the saturation capacities. Polymeric phases offer a higher density of interaction sites than monomeric phases (almost two times more) but their degree of heterogeneity is similar. Sites of type 2 account also for about 30 and 6% of the total saturation capacity for phenol and caffeine, respectively.

6.3. Monomeric endcapped packed C_{18}-bonded columns

Tables 3 and 4 give the best numerical values of the isotherm parameters found for phenol and caffeine on Kromasil, Symmetry, Luna, Hypurity and Discovery (all C_{18}-bonded, endcapped silica packed columns). The mobile phase was the same solution of methanol and water in all cases (30/70, v/v). All the adsorbents exhibit a bimodal AED for the adsorption of caffeine. Most exhibit also a bimodal AED for phenol, except for two columns, Symmetry and Discovery, for which the AED is trimodal, consistent with a tri-Langmuir isotherm model. The degree of heterogeneity of the adsorbent is higher with phenol than with caffeine. In two columns, a third type of adsorption sites was identified and the abundance of the type 2 sites relative to that of the type 1 sites is always larger with phenol (around 30%) than with caffeine (around 5%). Phenol seems to have access to numerous small hollow cavities inside the hydrophobic layer which caffeine cannot penetrate. It has been shown that the retention on the type 2 sites found on endcapped RPLC columns is entropically driven [69]. This explains the striking observation that the compound of the pair with the lower molecular mass (94 g/mol versus 194 g/mol) and the larger solubility in the mobile phase (>160 g/L versus <40 g/L) is the more retained. The reversed order of elution would have been expected in RPLC. This would take place if the high-energy sites were absent and the surface were homogeneous (the average Henry constant for all monomeric C_{18}-bonded columns would then be $H_1 = 1.0$ and 2.0 for phenol and caffeine, respectively). Because the surface is heterogeneous and type 2 sites are present, all predictions based on the hydrophocity and the solubility of the analyte are doomed to failure. The reason is that smaller solutes can penetrate more deeply inside the more accessible small cavities between the chains in the C_{18}-bonded layer than larger ones. On the other hand, as was expected, the average adsorption constants of caffeine on these phases are stronger than those of phenol ($b_1 \simeq 1.0$ and 2.5 L/mol and $b_2 \simeq 10$ and 25 L/mol for phenol and caffeine, respectively). The difference in retention does come from the different saturation capacites of the high-energy sites for the two compounds.

The separation mechanism of two simple analytes can be fully understood from the accurate knowledge of the parameters of their adsorption isotherms (i.e., the number of types of adsorption sites, their saturation capacities and equilibrium constants). The acquisition of this knowledge usually requires that isotherm data be measured at concentrations high enough to achieve the population of at least half the low-energy sites. Although half is still an empirical estimate, it is confirmed by our experimental observations. For example, in the case of the adsorption of phenol on Kromasil [85], convergence of the EM method in the calculation of the AED failed when FA data had been acquired with a maximum concentration of 50 g/L in the solution, leading to a surface coverages of 0.46 and 0.42 for the two highest data points. When additional measurements were made with concentrations up to 100 g/L, corresponding to surface coverages of 0.57 and 0.51 for the two highest data points, convergence of the AEDs was easily achieved, using the same number of energy grid points and the same number of iterations. By contrast, the simple injection of low concentration samples can only shed some light on the overall retention of the analyte, giving merely $\sum q_{S,i} b_i$. This number does not inform at all on the possible number of distinct types of adsorption sites on the surface, i, nor on the differences between their properties. As

a consequence, conclusions based on the mere consideration of retention factors, however big the data base used, are not meaningful.

6.4. "Supersites" of adsorption in RPLC

The adsorption behavior of the small analytes commonly used to study retention mechanisms is definetely not taken into account by a simple adsorption isotherm model assuming ideal adsorption on a homogeneous surface. That there are at least two different types of adsorption sites on RPLC surfaces is demonstrated by FA measurements. Table 7 lists the differences between the adsorption energies of the sites of types 2 and 1. Except for Discovery-C_{18} ($\Delta E \simeq 8$ kJ/mol for caffeine), these energy differences are strikingly close, whatever the brand of adsorbent considered, around 5–6 kJ/mol. Undoubtedly, these two types of sites are directly related to the C_{18}-bonded layer heterogeneity because of the very large number of these sites. For instance, Table 7 reports the surface concentration of the adsorbed analyte when the sites of type 2 are saturated. Using the small compound phenol, the surface concentration varies between 1 and 2 μmol/m^2. With caffeine, this concentration drops to between 0.05 and 0.20 μmol/m^2 because of the size exclusion of caffeine. Such a high surface concentration of phenol shows that type 2 sites cannot be residual silanols. There cannot be that many left after endcapping. A mixed retention mechanism occurs then between the solute and the bonded layer, whether with phenol, caffeine or any other small polar solutes, whose adsorption isotherm is convex upward (propranolol [45,128–131], nortryptiline [70], amytryptiline [71], naphthalene sulfonate [130]).

In addition to those sites on which the interaction of the surface with the analyte is based on dispersive interactions, higher energy sites can be encountered depending on the nature of the stationary phase, the mobile phase and the solute. These sites have a much lower density than the two sites aforementionned. Their adsorption energies are much higher (up to 20 kJ/mol) and it is not clear whether the corresponding solute-surface interactions are still based on dispersive interactions or are strong electrostatic interactions. Table 8 lists the different chromatographic systems that lead to at least three different types of adsorption sites. Note that for a RP-C_{30} stationary phase, the difference in adsorption energy between sites 2 and 1 is larger than with C_{18}-bonded phases (about 9 kJ/mol versus 5 kJ/mol). Typically, the adsorption energy is between 15 and 20 kJ/mol higher on sites of types 3 and 4 than on the lowest energy sites of type 1. These "supersites" may exist on conventional RPLC columns. As shown in Table 9, the concentration of sites of types 3 and 4 may vary between 5 and 700 nmol/L, and between 0.4 and 10 nmol/L, respectively. Some supersites of type 3 have a density close to that of type 2 adsorption sites. The solute-surface interactions on these sites involve dispersive interactions, the solute being probably entirely embedded in the C_{18}-bonded layer. All "supersites" of type 4 could reasonably be explained by the non-endcapped residual silanols that are embedded inside the bonded layer, based on their relative abundance and their adsorption energy. These sites deserve a particular attention when

Table 7
Adsorption energy difference between the sites of types 1 and 2 and surface density of adsorbed analytes when the sites 2 are saturated

Column (bonding process) MeOH/H_2O	Compound	$E_2 - E_1$ (kJ/mol)	Surface concentration sites 2 (μmol/m^2)	References
Hypersil C_{18} (monomeric) 30/70	Phenol	5.8	1.80	[69]
	Caffeine	5.4	0.13	
Kromasil C_{18} (monomeric) 30/70	Phenol	5.3	1.00	[69]
	Caffeine	5.5	0.07	
Phenomenex C_{18} (monomeric) 30/70	Phenol	5.6	1.04	[69]
	Caffeine	5.5	0.07	
Symmetry C_{18} (monomeric) 30/70	Phenol	5.9	0.83	[69]
	Caffeine	5.6	0.05	
Chromolith C_{18} 30/70	Phenol	5.5	1.10	[69]
	Caffeine	5.9	0.07	
Vydac C_{18} (polymeric) 30/70	Phenol	4.6	2.09	[69]
	Caffeine	7.3	0.14	
Xterra C_{18} (monomeric) 25/75	Phenol	7.6	1.85	[69]
	Caffeine	5.7	0.23	
Discovery C_{18} (monomeric) 30/70	Phenol	9.2	2.07	[113]
	Caffeine	8.0	0.05	
Resolve C_{18} (monomeric) 25 (MeOH)/75	Phenol	6.5	1.72	[45]
	Caffeine	5.4	0.35	
Gemini C_{18} (monomeric) 30/70	Phenol	4.9	1.09	[138]
	Caffeine	5.4	0.08	
Sunfire C_{18} (monomeric) 30/70	Phenol	5.0	0.73	[138]
	Caffeine	5.5	0.06	

Table 8
Adsorption energy difference between the sites of type 2, the "supersites" of types 3 and 4, and the lowest adsorption energy sites 1

Column (bonding process) organic/H_2O	Compound	$E_2 - E_1$ (kJ/mol)	$E_3 - E_1$ (kJ/mol)	$E_4 - E_1$ (kJ/mol)	References
Chromolith C_{18} 15 (MeOH)/85	Phenol	6.5	9.2	–	[78]
	Caffeine	4.3	7.5	13.5	
Symmetry C_{18} (monomeric) 20 (MeOH)/80	Phenol	6.7	11.2	–	[105]
Resolve C_{18} (monomeric) 25 (MeOH)/75	Caffeine	5.4	10.5	20.0	[45]
Discovery C_{18} (monomeric) 30 (MeOH)/70	Phenol	9.2	12.8	–	[113]
Prontosil C_{30} (polymeric) 30 (MeOH)/70	Phenol	7.0	15.6	–	[137]
	Caffeine	7.4	13.8	20.5	
	Propranolol	9.5	15.9	21.3	
	Naphthalene sulfonate	10.7	15.1	–	
Discovery C_{18} (monomeric) 28 (ACN)/72 phosphate buffer 20 mM, pH 2.7	Nortryptiline	14.1	18.3		[68,69]
	Amytryptiline	12.0	16.6		

the elution of positively charged analytes is attempted in the absence of buffer or of supporting salts that could otherwise be used to neutralize them.

RPLC adsorbents present on their surface low energy, dispersive sites and our measurements show that these sites are of two types. Their density depends on the size of the molecules of the analyte studied but remains of the order of a few μmol/m^2 for the sites of type 1 and between 0.1 and 1 μmol/m^2 for the sites of type 2. Higher energy sites or supersites are also detected and their density varies between 0.5 and 50 nmol/m^2. It is very likely that they are related to accessible silanol groups that remain after the C_{18} derivatization process. The adsorption energy on those supersites is systematically of the order of 20 kJ/mol higher than on type 1 sites. This energy is compatible with dipole–dipole interactions, hydrogen-bond interaction, and ionic exchange interactions.

6.5. Could the heterogeneity of RPLC adsorbents be controlled?

Manufacturers of columns and packing materials have made considerable progress since the inception of RPLC and are producing stationary phases that are far more reproducible now that 20 years ago. They keep investing and developing new columns and materials in a highly competitive market. However, they keep using commonly accepted tests to assess and prove the quality of their new products. Some of these tests remain highly relevant, for instances, those testing for the long-term stability of analytical retention in adverse conditions of temperature, pressure, pH, solvents or for the presence of heavy metals. The selectivity tests dealing with the hydrophobic and the steric selectivity, and with the retention of basic compounds certainly keep their usefulness. Many such tests are available [89,90] that allow manufacturers to guarantee, with a certain degree of confidence, that a good level of performance will be achieved. Things become more difficult when they want to prove that their columns do exhibit a very low degree of tailing, particularly for the elution of basic compounds. Most often, interactions with residual silanols, their high energy and the assumed correlative slow kinetics of desorption from the surface remain as they were 40 years ago, the weeping boys, the reasons given by conventional wisdom for the peak tailing of analytes. The essential heterogeneity of the C_{18}-bonded layer has remained ignored in spite of numerous experimental studies pointing the other way. The failure of silanol eradication to deliver phases on which basic compounds would not tail is illustrated by the properties of Xterra on which the density of silanols is extremely low. Yet our results demonstrate that this stationary phase is nearly as hetero-

Table 9
Surface densities of adsorbed analytes at the saturation of the "supersites" observed in RPLC

Column (bonding process) organic/H_2O	Compound	Surface concentration supersites 3 (nmol/m^2)	Surface concentration supersites 4 (nmol/m^2)	References
Chromolith C_{18} 15 (MeOH)/85	Phenol	350 (dispersive)	–	[78]
	Caffeine	230 (dispersive)	6(supersite)	
Symmetry C_{18} (monomeric) 20 (MeOH)/80	Phenol	690 (dispersive)	–	[105]
Discovery C_{18} (monomeric) 30 (MeOH)/70	Phenol	100 (dispersive)	–	[113]
Resolve C_{18} (monomeric) 25 (MeOH)/75	Caffeine	40 (supersite)	0.8(supersite)	[45]
Prontosil C_{30} (polymeric) 30 (MeOH)/70	Phenol	5 (supersite)	–	[137]
	Caffeine	7 (supersite)	0.43(supersite)	
	Propranolol	20 (supersite)	2(supersite)	
	Naphthalene sulfonate	17 (supersite)	–	
Discovery C_{18} (monomeric) 28 (ACN)/72 phosphate buffer 20 mM, pH 2.7	Nortryptiline	4 (supersite)	–	[70,71]
	Amytryptiline	11 (supersite)	–	

geneous than silica-based RPLC materials. If high-energy sites were entirely eradicated, the saturation capacity of the packing material would be a few hundred times higher, a blessing for analysts looking for trace compounds.

The in situ observation of the local heterogeneity of the surface, at the scale of the C_{18} chains could conceivably be made using modern mapping techniques (e.g., amplified force microscopy or AFM). This approach is made difficult by the roughness of the silica surface, down to the silicate ion level. Results obtained on silica plates are notoriously difficult to generalize to porous silica adsorbents. Solid-state NMR measurements were developed to study the heterogeneity of organic polymers and the relative mobility of their chains in the bulk [91]. This method has shed useful light on the chain conformation in the hydrophobic bonded layer and on the structural organization of these chains [92]. Albert et al. has shown that, typically, the alkyl chains assemble in domains in which they have one of two main arrangements, one of high relative mobility (mainly made of chains in the gauche conformation), and one of low relative mobility (mainly made of alkyl chains in the trans conformation) [93–95]. Fluorescence spectroscopy provides another sensitive approach to assess the effect of the bonded phase structure on the partitioning and the mobility of analytes [95]. It was shown that the higher the water content in acetonitrile:water mixtures, the longer the fluorescence lifetime of 1,6-diphenylhexatriene (DPH) embedded within various C_{22}-bonded stationary phases and the lower its mobility. This work provides little direct information on the nature of the adsorption sites in RPLC packing materials. Yet, by pointing out the coexistence in the bonded layer of random, fluid domains and of quasi-crystallized, rigid domains, this work justifies our suggestion made earlier of the high-energy sites being cavities inside the bonded layer in which all van der Waals interactions would be more intense than at the interface with the solution.

The results of systematic investigations using spectroscopic or chromatographic methods seem to agree on the high degree of heterogeneity of the hydrocarbon bonded layer. The more rigid and the more mobile domains revealed by NMR measurements could correspond to the adsorption sites of type 1 and the partition sites of type 2 (and possibly those of types 3 and 4 when these ones exist) that are observed on the commercial C_{18}-bonded phases through the measurements of FA adsorption data, the modeling of these data, and the calculation of the AED. A combination of cross-polarization magic-angle-spinning (CP/MAS) NMR, of two dimensional solid-state NMR spectroscopy (wide line separation, WISE) with FA measurements and the calculation of AEDs should provide manufacturers with the tools that they need to characterize their old and new packing materials. These techniques could be most useful at following and quantifying accurately the improvements made in the degree of surface heterogeneity of these materials, depending on the changes made in their preparation process.

Peak tailing is not necessarily the result of the presence of residual silanols on the surface and of nothing else. It is not necessarily a kinetic effect; it can have and, most probably, it often has a thermodynamic origin. It does not concern only basic compounds. In consequence, manufacturers have to face new and different challenges to improve the methods of preparation of hydrophobic surfaces. Different compromises will have to be found between the degree of surface heterogeneity of new materials and their various chromatographic properties (e.g., retention, selectivity, efficiency, peak asymmetry at low concentration). A completely homogeneous support may not be the chromatographic material performing best under all conditions. Yet, detailed investigations of the separation of amytriptylline and nortriptyline illustrates how much the heterogeneity of the bonded layer of alkyl chains impair the performance of analytical and preparative chromatography alike [70].

7. Experimental conditions and adsorption isotherms of neutral compounds

Analytical chemists are always looking for better optimization of the experimental conditions of their separations. In analysis, optimization is the search of an optimum compromise between a complete resolution of the compounds of interest and a short analysis time. One approach to do that consists in acquiring the smallest possible amount of experimental data that span the useful part of the available range of the experimental parameters involved. Then, using a dedicated chromatographic software, the experimental parameters are optimized to satisfy the objective function selected. A profoundly different approach consists in understanding the fundamental effects of the variations of each experimental parameter on the equilibrium isotherm. This method provides the analyst with the possibility to predict accurately the changes caused to a separation by an adjustment of the experimental parameters. The advantage of the latter method over the former one is that it does not blindly ignore the fundamentals of adsorption. Temperature, average column pressure, mobile phase composition, and the nature of the organic modifier used in RPLC are usual experimental parameters that chromatographers routinely modify. The impact of these changes on the retention and separation of analytes in RPLC can be investigated easily.

We discuss in this section the influence on adsorption data measured by FA of changes in the experimental parameters. The study of these changes provides useful conclusions regarding the relationship between adsorption isotherms and retention mechanisms.

7.1. Effect of pressure

The pressure or the average column pressure is a parameter that chromatographers usually do not care much about. They ignore that pressure has an effect on the equilibrium between the mobile and the stationary phases. Furthermore, until recently, they could not control it because detector cells could not withstand any significant pressure. Now, many do. However, the average column pressure depends on the flow rate applied, on the temperature (through the viscosity of the mobile phase), and on the permeability of the column. Hence, pressure is often not considered as an experimental parameter. Actually, however, a correct isotherm determination requires that the average column pressure be known [96]. Because the effects of pressure are lin-

ear, the isotherm measured for a given average column pressure is identical to the isotherm measured at a constant pressure equal to that average pressure.

Classical thermodynamics [97,98] provide a simple relationship between the retention factor k' and the local pressure, at constant temperature of the system:

$$\left(\frac{\partial \ln k'}{\partial P}\right)_T = -\frac{\Delta V}{RT} + \left(\frac{\partial \ln \phi}{\partial P}\right)_T \quad (26)$$

where ΔV is the difference between the partial molar volumes of the solute in the two phases, ϕ the column phase ratio, and T is the temperature.

In most cases in RPLC, ΔV is negative, the partial molar volumes of the solute in the stationary phase is smaller than in the mobile phase, and the retention factor increases with increasing pressure. The fact that ΔV is negative means that the conformation of analytes is more compact in the hydrophobic environment of the bonded layer than in the mobile phase. It is important to underline that in Eq. (26), it is the absolute, not the relative, molar volume change associated with the equilibrium that figures in the relationship. The higher the molecular mass of the analyte, the larger its molar volume, hence, the larger ΔV (in broad general terms; minor deviations are possible). For instance, ΔV is of the order of -10, -50 and -100 mL/mole with low-molecular-mass molecules such as phenol derivatives [99] or thiourea used as a dead column tracer [43], with medium molecular-mass peptides such as bradykinin and kallidin [100], and with macrobiomolecules such as insulin [101–103], respectively.

Liu et al. [103] measured the influence of the average column pressure on the isotherm of insulin on a C_8-bonded silica stationary phase. These authors found that the isotherms fit well to a Langmuir model, suggesting that molecules of the size of insulin ($M_W \simeq 6000$ Dalton) do not see the adsorbent surface as heterogeneous. Interestingly for preparative chromatography, the saturation capacity of the column increases significantly with increasing average column pressure (by ca. 20% for an increase of 200 bar. This could provide a useful (but costly) increase in the production rate.

Most importantly, these few examples demonstrate the necessity of measuring the adsorption isotherms at a controlled average column pressure, at least for large molecules. Very little research work has been carried out so far, however, to assess the effect of ultra-high pressures (up to a few kilobar) on retention mechanisms in RPLC. Increasing the pressure from 0 to 200 bars typically increases the retention factors and the column saturation capacity by 100 and 20%, respectively, for biomolecules. Since the effects of pressure tend to be linear in the range up to a few kilobar, it should be no surprise if retention factors, volumes, and times, resolution and even retention patterns of biomolecules are widely affected by a change in mobile phase flow rate. While those who keep ignoring pressure effects on retention mechanisms will keep getting confusing results and unexpected difficulties in method development, those knowledgeable in physical chemistry will have great opportunities. Much work remains to be done in this area.

7.2. Effect of temperature

The effect of temperature on the distribution of analytes at infinite dilution between the stationary and the liquid phases is well known. The Van't Hoff equation relates the retention factor and the temperature:

$$\ln k' = -\frac{\Delta H}{RT} + \frac{\Delta S}{R} + \ln \phi \quad (27)$$

Obviously, the Van't Hoff equation assumes that there is a single adsorption mechanism (hence that the surface is homogeneous). The plot of $\ln k'$ versus $1/T$ is linear only if the associated thermodynamic parameters, ΔH and ΔS, are invariant with temperature changes. However, these assumptions are not likely to be fulfilled in RPLC. First, it was shown that these stationary phases are heterogeneous (see Section 7). Secondly, it is known that the structure of the alkyl bonded chains changes somewhat with temperature. Despite this, a majority of the plots of $\ln k'$ versus $1/T$ found in the literature are linear or nearly so. This may be because the temperature ranges investigated are not wide enough, given the relatively small difference between the values of ΔH corresponding to the sites of types 1 and 2.

Although a considerable amount of data is available in linear chromatography, few data have been published in nonlinear chromatography, so we do not have much information to assess the temperature influence on the saturation capacities and the equilibrium constant of adsorption isotherms. A preliminary study has recently investigated the temperature influence on the adsorption isotherm of phenol on Kromasil-C_{18} [104]. Useful qualitative information was derived but more data need to be acquired to allow the drawing of general conclusions. As shown earlier in this review, the adsorption isotherms of phenol on Kromasil are best described by a bi-Langmuir isotherm (with four parameters, $q_{S,1}$, b_1, $q_{S,2}$, and b_2). The FA isotherm data lead to a saturation capacity $q_{S,1}$ (low-energy sites) that decreases by a factor 2 when the temperature increases from 298 to 358 K. In contrast, the equilibrium constant b_1 was little affected by this 50 °C change. Conversely, the saturation capacity of the high-energy type of adsorption sites, $q_{S,2}$, remains practically constant in this temperature range while the equilibrium constant, b_2, drops by a factor 4. This suggests that the structure of the first type of sites, the top of the C_{18}-bonded chains, is not affected by temperature. b_1 remains constant probably because of a compensation between the classical temperature effect (see Eq. (8)) and the increase in contact area between the increasingly disordered chains and the analyte. The number of high energy sites is poorly affected, so b_2 experiences the expected decrease with increasing temperature because the surface area of contact between the analyte and the hydrophobic layer cannot increase. An adsorption energy of about 15 kJ/mol was derived on the sites of type 2.

Another study quantified the effect of temperature on the equilibrium constant of the analyte on the highest energy sites of Symmetry-C_{18} in a methanol:water solution (25/75, v/v) [105]. Fig. 21A shows the evolution of the overloaded band profiles of phenol on Symmetry with increasing temperature. The best values of the equilibrium constants were derived for each tem-

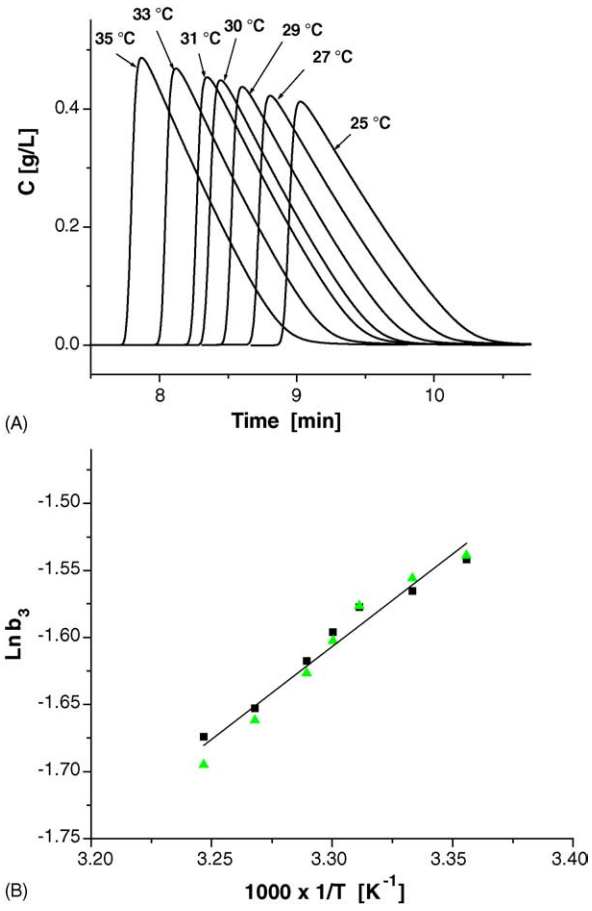


Fig. 21. (A) Overloaded band profiles of phenol recorded on the Symmetry-C_{18} column for seven different temperatures. Methanol:water, 20/80 (v/v). (B) Evolution of the best estimated equilibrium constant of the highest energy site (b_3) vs. the reciprocal temperature from low (triangles) and high (squares) loading bands. Same experimental conditions as in (A). Note the linearity of the plot in agreement with the classical Van't Hoff law. The derived adsorption energy ϵ_3 is 11.5 kJ/mol, a typical enerfy for hydrophobic interactions. Reproduced with permission from [105] (Figs. 3A and 4).

perature, using the inverse method of isotherm determination. The plot of the logarithm of the adsorption constant on the highest energy sites (type 3), b_3, versus the reciprocal of the temperature is linear and follows Eq. (8) (Fig. 21B). This result permits the determination of the absolute adsorption energy, ϵ_3, on these sites. It is 11.5 kJ/mol larger than ϵ_1 on Symmetry. This energy is still well in the range of hydrophobic interactions and is markedly too small to correspond either to the formation of hydrogen-bonding or to dipole–dipole interactions (for which $\Delta H \simeq$ 20–25 kJ/mol). As suggested in Section 6 on the heterogeneity of modern RPLC columns, the high-energy sites cannot be correlated to any interactions taking place between residual silanols and the analyte. As expected, the density of the high-energy adsorption sites is not much affected by temperature. It decreases only slightly, at a rate of −0.26 g of phenol per liter of Symmetry adsorbent per °C. Typically, an error in the temperature of the order of 1 °C affects the equilibrium constant and the saturation capacity of a low-molecular mass compound on the high-energy sites by about 1.5% [105].

7.3. Effect of the mobile phase composition

There are few examples demonstrating the effect of changes in the mobile phase composition on the values of the parameters of the adsorption isotherm [106,107,85]. Quantitative information has been derived from the adsorption behavior of phenol on Kromasil from aqueous solutions of methanol having from 0 to 60% methanol, v/v [85,86]. The variations of the best parameters of the bi-Langmuir model are shown in Fig. 22. As suggested by the linear solvation strength model (LSSM), the equilibrium constants b_1 and b_2 decrease with increasing methanol concentration and their logarithms are linear functions of the molar fraction φ of methanol in the mobile phase. The evolution of the density of adsorption sites available for phenol is informative. The quantity of low energy sites in the column remains nearly constant while that of the high energy sites decreases significantly with increasing methanol concentration. Because the C_{18}-bonded chains are more easily solvated at high methanol concentrations than in a water-rich mobile phase, the bonded layer swells and gets more disorganized in contact with a water-rich mobile phase. The gauche conformation of the C_{18} chains becomes more predominant and additional hydrophobic cavities are formed in the hydrophobic layer. Conversely, when the methanol concentration in the mobile phase exceeds 70%, a majority of the alkyl chains are in the all-trans conformation, the high-energy sites tend to disappear, and Kromasil-C_{18} behaves as an adsorbent with a nearly homogeneous surface. This phenomenon seems rather general; for instances, Dorsey et al. [108] and Carr et al. [109] argued that the use of 3% *n*-propranol as a constant component of the mobile phase virtually eliminates reequilibration following gradient elution. One possible interpretation of this observation is that most of the high-energy sites are blocked by this solvent.

In order to confirm these observations regarding the evolution of the isotherm parameters when the mobile phase composition changes, gradient elution chromatography was performed on the same column and with the same analyte [87]. The resulting overloaded band profiles were compared to those calculated from the ED model, with the isotherm model obtained. Consistent with the experimental results presented above, it was assumed that the saturation capacity $q_{S,1}$ remained constant, that $q_{S,2}$ decreased linearly with increasing methanol fraction in the mobile phase, φ, and that the equilibrium constants b_1 and b_2 followed the LSSM behavior. The general adsorption isotherm is written as follows

$$q^*(C,\varphi) = q_{1,0}\frac{b_{1,0}\exp(-S_{b_1}\varphi)C}{1+b_{1,0}\exp(-S_{b_1}\varphi)C} + (q_{2,0}-S_{q_2}\varphi)\frac{b_{2,0}\exp(-S_{b_2}\varphi)C}{1+b_{2,0}\exp(-S_{b_2}\varphi)C} \quad (28)$$

where the subcripts 1 and 2 refer to the low- and the high-energy sites, respectively, $q_{1,0}$ is a constant, $b_{1,0}$ and S_{b_1} are the intercept and the slope of the plot of $\ln(b_1)$ versus the methanol fraction, φ, respectively, $q_{2,0}$ and S_{q_2} are the intercept and the slope of the plot of $q_{S,2}$ versus φ, respectively, and $b_{2,0}$ and S_{b_2} are the intercept and the slope of the plot of $\ln(b_2)$ versus φ, respectively. The

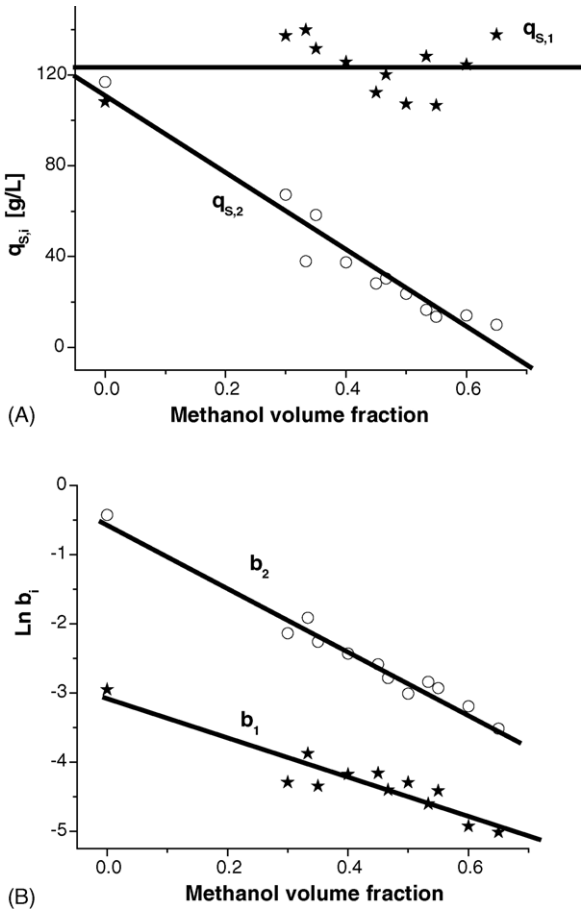


Fig. 22. Evolution of the best isotherm parameters of phenol, (A) saturation capacities, (B) equilibrium constants, on the Kromasil-C_{18} adsorbent vs. the methanol content in the mobile phase. The parameters were derived from FA measurement, except for the mobile phase with pure water (FACP). Note that the equilibrium constants follow well the LSSM model and the saturation capacity of the high energy sites decreases rapidly with the methanol content. Reproduced with permission from [87] (Fig. 1).

best values of the numerical parameters of this general isotherm are given in ref. [87].

Fig. 23 shows the excellent agreement observed between the calculated and experimental band profiles. This agreement confirms the validity of the adsorption isotherm model and supports the mechanism of phenol adsorption on C_{18}-bonded phases that is proposed.

The concentration of the organic modifier affects the degree of heterogeneity of reversed-phase stationary phases. This demonstrates that the organic modifier affects the structure of the bonded alkyl layer and that the changes in the structure of this layer affect the properties, and particularly the density, of the high-energy adsorption sites. When the mobile phase has a high water concentration, the retention factors of analytes increase because their solubility decreases and because the density of high-energy sites increases. Furthermore, this increase is accompanies by an aggravation of the degree of tailing of the bands that are overloaded for lower and lower sample amounts [85]. The apparition of new, higher energy adsorption sites is sometimes the consequence of the use of a mobile phase having a

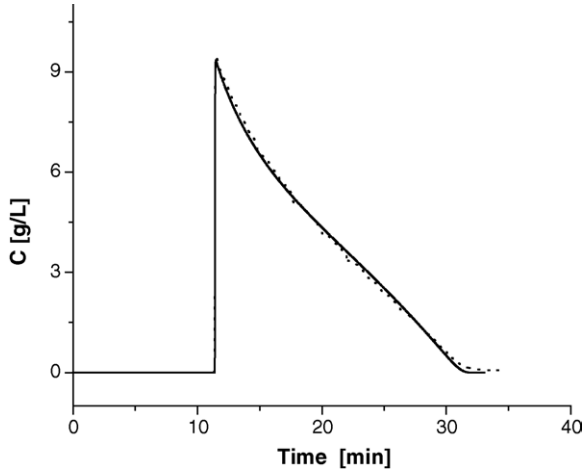


Fig. 23. Validation of the general adsorption isotherm with the mobile phase composition by comparing experimental and simulated band profiles acquired under gradient elution conditions. Injection of a 40 g/L solution of phenol dissolved in pure water during 2 min. Flow rate, 1 mL/min; gradient times, 40 min; gradient step, 0–50% methanol. Reproduced with permission from [87] (Fig. 8).

high water content. This effect was observed on Symmetry. The AED of phenol is bimodal and its isotherm follows bi-Langmuir isotherm behavior with 30% methanol [69] but the AED becomes trimodal and the isotherm a tri-Langmuir one with 20% methanol [105].

7.4. Effect of the nature of the organic modifier

Organic modifiers are used to adjust the solubility of the sample components in the mobile phase. They are classified according to their strength. When the elution time of an analyte is too long, it can be reduced either by increasing the concentration of the organic modifier or by replacing it with a stronger one. For instance, isopropanol is stronger than ethanol, which is stronger than methanol. Acetonitrile is known to be a stronger eluent than methanol. It is often used to elute large molecular-size compounds such as biomolecules. Still, results on the relative strength of organic modifiers are based on data collected upon the injection of infinitesimal amounts of probe analytes and are related to linear chromatography. These data inform on the apparent equilibrium constant between the two phases of the chromatographic system. They are silent regarding the saturation capacities. There is practically no data available in the literature which compares the isotherms of a compound on the same C_{18}-bonded stationary phase with methanol:water and acetonitrile:water mobile phases.

Acetonitrile and methanol have quite different physicochemical properties. The structures of their aqueous solutions differ markedly. Among other differences, methanol and water molecules associate readily through hydrogen-bonding interactions [110] while acetonitrile molecules form clusters with other acetonitrile molecules and interact little with water molecules. The adsorption behavior of acetonitrile and methanol on RPLC materials is also fundamentally different. The measurement of the excess isotherms of methanol and acetonitrile on C_{18}-bonded

phases has shown that the former forms an adsorbed monolayer while the second forms a multilayer adsorbed system containing usually 3 to 5 monolayers. Accordingly, the pure acetonitrile multilayer at the surface of RPLC adsorbents is considered by certain authors as a "third phase," in equilibrium with the bulk mobile phase (acetonitrile:water) and the C_{18}-bonded layer. According to these authors, the solutes would take part into two equilibria, one between the bulk mobile phase and the adsorbed layer of pure acetonitrile, the other between this acetonitrile layer and the C_{18} chains. Based on this description of the equilibrium system, the retention factors of neutral compounds [111] and of ions [112] were predicted and the predicted values match well with the experimental data.

A recent study demonstrated that the adsorption behavior of neutral compounds on the same RPLC columns differs fundamentally whether methanol or acetonitrile are used as the organic modifier [113]. While the curvatures of the isotherms of phenol and caffeine are always convex upward or langmuirian with methanol, the shape of these isotherms changes at high solute concentrations when the organic modifier is acetonitrile. The best adsorption models were a bi- and a tri-Langmuir isotherm for phenol and caffeine with methanol (isotherm of group I), and the sum of a Langmuir and a BET isotherm models (isotherm of group I + isotherm of group II) with acetonitrile (See Fig. 24).

It is striking to observe that the amount of solute adsorbed from a solution of given concentration is lower at low concentrations with an acetonitrile-based than with a methanol-based mobile phase (consistent with the solute being eluted earlier with acetonitrile) and higher at high concentrations. In other words, the adsorption isotherms from methanol and from acetonitrile solutions cross each other. Accordingly, it makes no sense to say that acetonitrile is a stronger eluent than methanol. What is true at low solute concentrations is false at high concentrations. We suggest that, at low concentrations of the solute, its retention depends essentially on its distribution between the adsorption sites that are located within the C_{18}-bonded layer (and constitute the highest energy sites) and the other two phases, the aqueous methanol bulk phase and the adsorbed layers of acetonitrile or "third phase". In this range, the isotherm is convex upward. At high concentrations, when the adsorption sites are saturated, the solute accumulates in the adsorbed multilayer of acetonitrile where its concentration increases faster than in the mobile phase. The isotherm becomes convex downward.

The nature of the organic modifier affects considerably the composition of the interface layer and has profound consequences on the adsorption mechanism of analytes. Because the organic modifier may accumulate on the C_{18}-bonded phase, the interface between the bonded chains and the bulk mobile phase has a composition and a dimension that may differ markedly from one modifier to the next. This interface controls the amount of compound that can be adsorbed and may change considerably the saturation capacity of a column. More data are required, particularly data acquired with other organic modifiers (e.g., isopropanol, tetrahydrofuran) to draw more general conclusions regarding the effect of the organic modifier on the adsorption mechanisms in RP-HPLC.

8. Experimental conditions and adsorption isotherms of ionizable compounds

The analysis of ionizable compounds is of great importance in the biochemical, biomedical, pharmaceutical, and environmental fields. RPLC is the method most frequently applied to purify and analyze this class of compounds. It requires the selection of an organic modifier and of either supporting salts or buffers, in order to fix the pH and the ionic strength of the mobile phase. While we understand now rather well the thermodynamics of phase equilibria involved in the separation and purification of neutral compounds in RPLC, the same is not true yet for ionizable compounds. Two different types of mechanisms are currently proposed in the literature.

- (1) A first mechanism is based on an electrostatic description of the equilibrium system. The formation of a double layer at the solid–liquid interface [114] and repulsive interactions between the charged surface (zeta potential) and the analyte [115–117] are proposed to account for the adsorption of ions. In these electrostatic models, the ionic strength of the solution is a critical parameter, not the nature of the supporting salts or of the buffer.
- (2) A second type of mechanism takes into account the possibility for ions to be involved in an association equilibrium with any counter-ion present in the solution [118–121]. Ion-pairing may take place in the mobile phase and the adsorption behavior of one particular ion will depend on the adsorption equilibrium of all the forms in which it may be involved (either free or as ion-pair complexes) on the solid surface. Because the molecular exchange between the free solvated ion and the complexes in which the ion may be involved is extremely fast compared to the characteristic time of mass transfers in chromatography, only one peak or overloaded band is observed. The problem can then be treated as for neutral compounds, provided one keeps in mind that the saturation capacities and the binding constants derived from FA data are actually values averaged over the contributions of all the species (free and complexed ions) in which the compound studied is involved. Under these assumptions, the ionic strength and the nature of the buffer or salt molecules are critical factors in the adsorption mechanism of ionizable compounds. The special case of the adsorption of ionizable compounds in the absence of buffer or supporting salt will also be discussed.

8.1. Absence of buffer and supporting salts

It is uncommon to elute ionizable compounds with a mobile phase containing neither a salt nor a buffer. Although this is generally not a recommended procedure, it can be done and it provides useful clues in the study of retention mechanisms. Adsorption isotherms of ionizable compounds can be measured in neat mobile phases as long as the properties of the mobile and the stationary phases are not drastically affected by changes in the concentration of the studied compound.

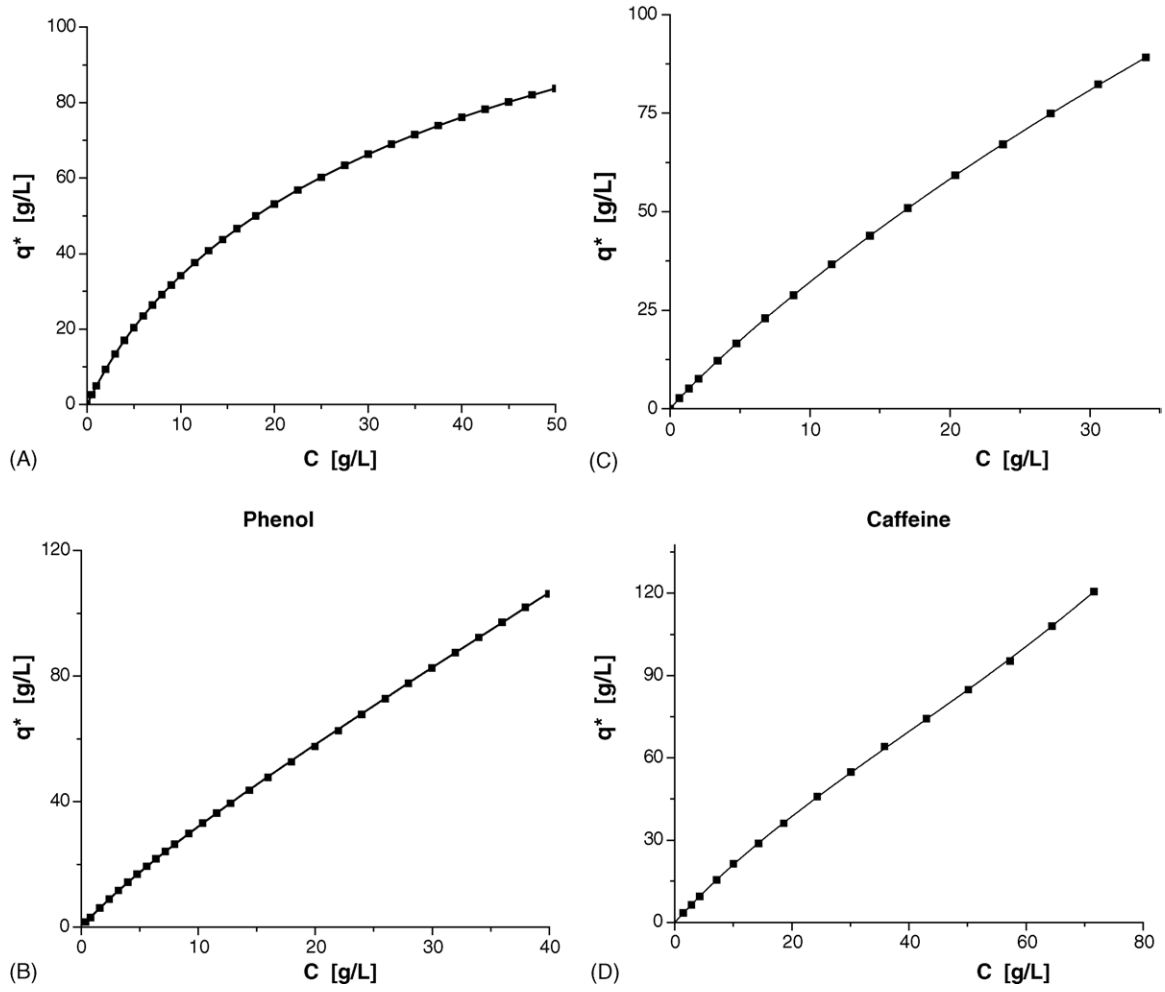


Fig. 24. Adsorption isotherms of phenol on Kromasil-C_{18} using a methanol:water (A; 40/60, v/v) and an acetonitrile:water (B; 30/70, v/v) as the mobile phase. Adsorption isotherms of caffeine on Discovery-C_{18} using a methanol:water (C; 30/70, v/v) and an acetonitrile:water (D; 20/80, v/v). $T = 295$ K. Note that the curvature of the isotherm is reversed at high concentrations when acetonitrile is used as the organic modifier. Reproduced with permission from [113] (Figs. 1A, 3A, 11A, and 12A for Fig. 24A–D, respectively) ©2005, American Chemical Society.

For instance, propranololium chloride (propranolol is an amino alcohol used as a β-blocker) can be dissolved in an aqueous methanol solution, at concentrations up to 40 g/L. Its adsorption isotherm was measured by FA on Kromasil-C_{18} [122]. The isotherm obtained is S-shaped (convex downward at low concentrations, upward at high concentrations). In contrast, the isotherm of propranolol is strictly convex upward on the same stationary phase but with a mobile phase containing a buffer, as reported in the literature [123]. An isotherm model of our group I (see Eq. (23)), the bi-Moreau isotherm, was found best to account for the adsorption data and to predict overloaded band profiles in close agreement with the experimental ones. In other words, a monolayer of propranolol is formed on the surface, with an adsorbate–adsorbate interaction energy between two and three times *RT*. These interactions vanish when a 0.2 M acetate buffer is added to the mobile phase and the best isotherm becomes the classical bi-Langmuir model. Surprisingly, the saturation capacity of Kromasil-C_{18} is nearly the same whether the mobile phase contains a buffer or not.

This result contradicts other findings showing that the saturation capacities of ionizable compounds are at least one order of magnitude lower than those of neutral compounds [124]. The contradiction stems from two different reasons. First, like most chromatographers, McCalley draw his conclusions regarding the column saturation capacity from plots of the apparent efficiency of overloaded band profiles or of breakthrough curves versus increasing concentrations of the solute. Second, the influence of the surface heterogeneity of the adsorbent in the column, of the existence of few high-energy adsorption sites on this surface, and the contributions of this heterogeneity to the band profiles is ignored.

This has led to contradictory results regarding the determination of the column saturation capacity of an ionizable compond on RPLC columns [70]. When slightly overloaded band profiles are recorded, as it was done by McCaley [124], only the high energy sites are filled while the low energy sites (type 1) remain almost empty. The estimate of the saturation capacity measured under these conditions is simply a fair estimate of the saturation capacity of the high-energy sites. Using this method,

McCalley found that the saturation capacity of nortrypytiline on Discovery-C_{18} is of the order of one mg/g of adsorbent. Using the FA method, we determined that the overall saturation capacity of the same column, for the same chromatographic system is of the order of 100 mg/g, hence one hundred times larger. Such a large factor needed definitely some clarification. The results derived from the FA method confirmed that the adsorption of nortryptyline could only be modeled by a tri-langmuir adsorption isotherm model. The saturation capacity measured by the former method corresponds to the sum of the saturation capacities of the second and third types of sites. The overall saturation capacity measured by the latter method is the sum of the capacities of the three types of sites.

As a consequence, the data obtained are actually a fair estimate of the saturation capacity of the high-energy sites. Admittedly, this is the important characteristic for analytical applications. However, linear chromatography provided no information regarding the total saturation capacity of the adsorbent (i.e., the sum of the saturation capacities of the high- and low-energy sites) since the low-energy sites are not populated during the series of measurements performed and the estimate obtained for the total column saturation capacity is incorrect. The fact that neutral and ionizable compounds lead to comparable values of the total column saturation capacity is due to the adsorption of ions as neutral ion-pair. In the case of propranololium, it was demonstrated that this cation does adsorb with his co-ion chloride [125]. The higher the concentration of chloride in the solution, the higher the retention of propranolol because the proportion of free, solvated and poorly retained free ionic species decreases with increasing $[Cl^-]$. Accordingly, the absence of buffer or supporting salt decreases the hydrophobity of the compound and some mobile phase adjustments are required to achieve comparable retention times.

The adsorption isotherms of nortriptyline and amytriptyline chloride (positively charged ions, used as antidepressors) were also measured without buffer or supporting salts in the mobile phase on a Discovery-C_{18} column [70,71]. They both exhibit the same characteristics as propranolol, e.g., adsorbate–adsorbate interactions take place in the stationary phase and a tri-Moreau model applies in all these cases. Again, the heterogeneity of the column is obvious with most of the adsorption sites being low-energy sites (98%) and very few high-energy sites (2%) being available.

From a more practical point of view, it is possible to predict the overloaded band behavior of ionizable compound with no buffer nor salt in the mobile phase in RPLC. As for neutral compounds, their isotherm can be accurately measured by FA (except at very low concentrations) and modeled with one of the usual isotherm models. Highly concentrated solutions of ionizable molecules can be prepared with the addition of other chemicals in the mobile phase, which avoids the cumbersome procedures required to remove the excess salt or buffer.

8.2. Adsorption isotherm and pH

Whether the acidic or the basic forms of an ionizable compound has to be analyzed, any one of these forms can be selected by adjusting the pH of the solution. Neue et al. [126] showed that suitable manipulations of the pH can enhance the column loadability by a factor up to 20. The difference was attributed to the difference in the ionization states of the analyte. These authors observed that the departure of the isotherm from linear behavior was more important and began at lower concentrations for the ionic than for the neutral species. Based on this result, they concluded that the ionic form has a much lower saturation capacity than the neutral one. However, they did not take into account the surface heterogeneity and the presence of different types of sites, particularly of high-energy sites with a very low saturation capacity. These sites are responsible for the nonlinear behavior of the isotherm at very low concentrations. The much lower saturation capacity of high-energy sites for ionic species is consistent with the total capacity of the column being about constant. Unfortunately, for chromatography, the low saturation capacity of the sites of highest energy sets the maximum sample size that can be used.

The solubility of an ionic species being higher in aqueous mobile phases than that of the corresponding neutral species, it is less strongly retained and the pH can be used to control the retention of compounds with an acidic and a basic form. To obtain

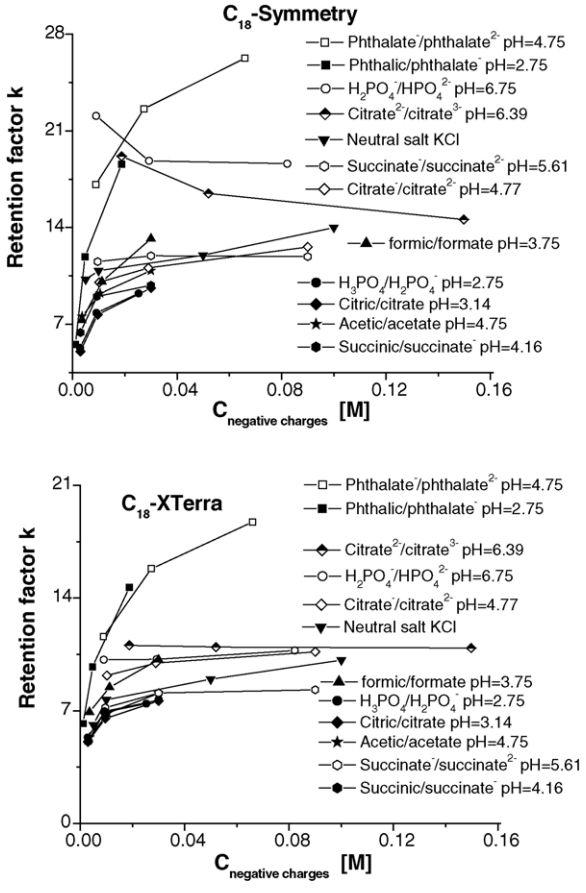


Fig. 25. Evolution of the retention factor of propranolol as a function of the concentration of negative charges coming from the buffer anions on the Symmetry and XTerra columns, $T = 295$ K. Note the influence of the nature of the buffer for a same concentration of negative charges and the non-dependence of the retention factor on the pH of the mobile phase. Reproduced with permission from [134] (Fig. 1).

comparable retention factors for the two forms, the content of the organic modifier is usually 10–20% higher for the elution of the neutral form than for that of the ionic form [124,127]. Typically, the ratio of the retention factors of the acidic and the basic forms of a compound is of the order of 10 [127]. When plotted as a function of the pH, the retention factor of an acido-basic compounds vary sharply around the pK_a (i.e., between $pK_a \pm 2$ pH units). Outside this pH domain, the retention of ionizable compounds is nearly independent of the pH and depends only on the nature of the buffer used when the ionic form of the compound forms an ion-pair with one of the buffer ions. This effect is illustrated in Fig. 25 which shows the evolution of the retention factor of propranolol ($pK_a \simeq 9.2$) as a function of the concentration of different buffer systems. The pH range investigated

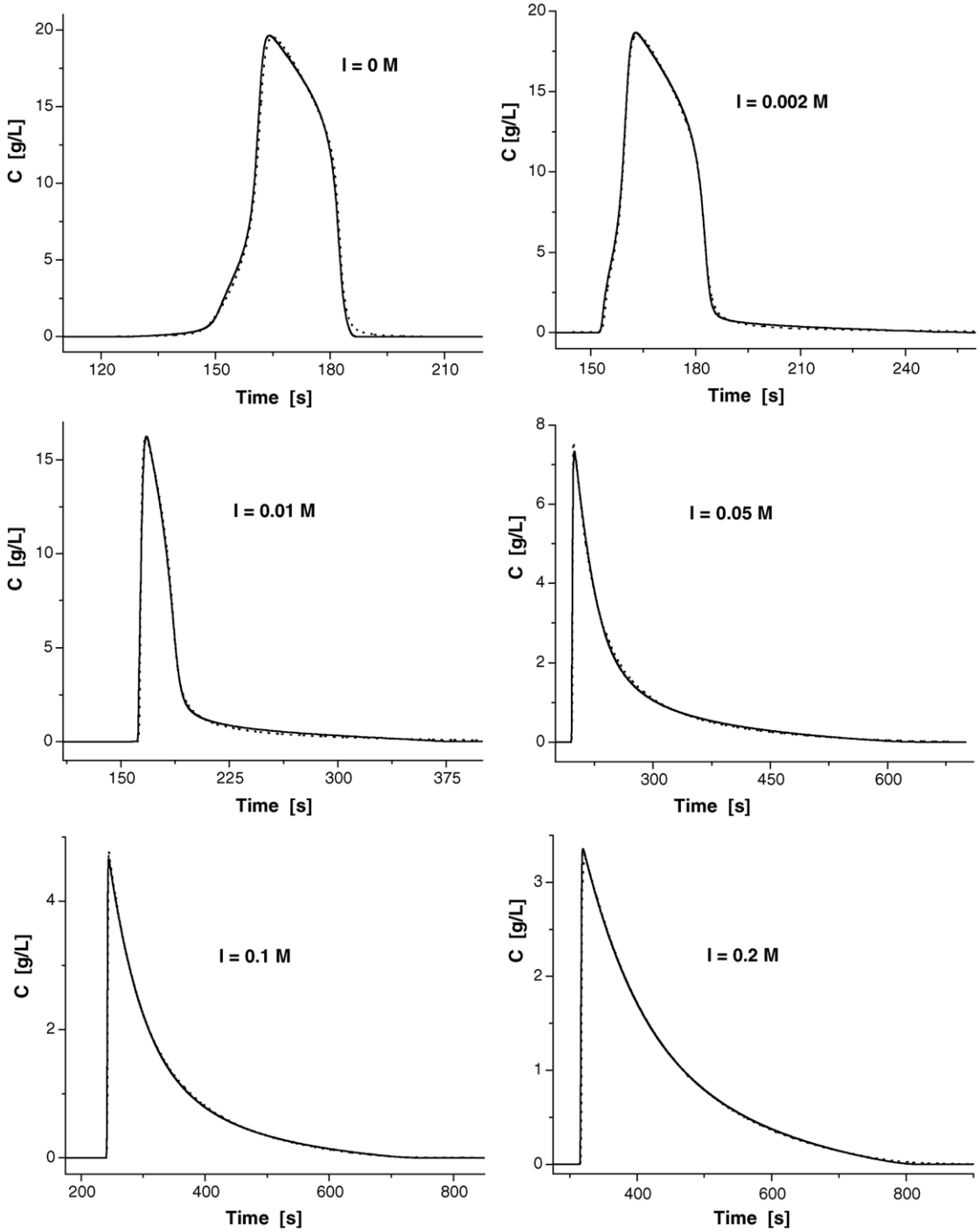


Fig. 26. Evolution of the shape of the overloaded band profile of propranolol as a function of the concentration of supporting salts (KCl) in the mobile phase (methanol:water, 40/60, v/v) on the XTerra column. Flow rate, 1 mL/min; $T = 295$ K. Note the progressive "langmuirization" of the isotherm suggested by the formation of a front shock and rear tailing at high salt concentrations. Reproduced with permission from [131] (Fig. 4A) ©2004, American Chemical Society.

was between 2.5 and 6.5. Because the pK_a of this compound is much higher than the pH range investigated, no change should be expected in the retention factor of the ion. Yet, the experimental results show that the retention factor significantly varies from one to another buffer (e.g., phthalate buffer compared to other buffers) and that there is no correlation between the pH and the retention factor. Instead, the nature of the buffer and the hydrophobicity of its anion control the retention, based on the formation of ion-pairs.

8.3. Adsorption isotherm and ionic strength

The adsorption isotherms that best model the behavior of an ionizable compound in a chromatographic system with and without a buffer in the mobile phase are fundamentally different. In the former case, a multi-Moreau isotherm including adsorbate–adsorbate interactions applies. In the latter case, a simple multi-Langmuir isotherm with no lateral interactions describes well the adsorption behavior of the ion. A progressive increase of the ionic strength of the mobile phase affects the adsorption behavior. Experimental results showed that there is a link between the two extreme situations (i.e., the absence and the presence of electrolytes in the mobile phase) [128–131]. As the concentration of the supporting salt is increased, the adsorbate–adsorbate interactions progressively vanish and the shape of the experimental overloaded band profiles evolves accordingly (Fig. 26). The inverse method for isotherm determination was used to extract the best numerical values of the parameters of the bi-Moreau isotherm model in the case of propranolol [128–131]. The results confirmed this trend. Increasing the salt concentration leads to an increase of the equilibrium constant on the low-energy sites and to a decrease of the equilibrium constant on the high energy sites. An increase of the two saturation capacities with increasing ionic strength is also observed.

As a general rule, an increase of the ionic strength of the solution leads to a shift of the elution band toward higher retention times and to a significant broadening of the band (Fig. 27). This is true for anions and for cations [132]. In both cases, this fact suggests the formation of neutral ion-pair complexes, which become more abundant at high salt concentrations (according to the equilibrium constant between the ion-pair and the free ion or common ion effect). Combining the effects of the ionic strength and of the organic modifier concentration allows an easy adjustment of the retention of ionizable compounds.

8.4. Adsorption isotherm and buffer valence

When the pH differs much from the pK_a of the analyte, changing it has little influence on the adsorption of ionizable compounds. The concentration of the buffer, however, affects markedly the adsorption of ions. Yet, no systematic studies have demonstrated or even mentioned the role played by the valence of the buffer. The main reason for that is that most studies using buffers are dealing with linear chromatography. Recent studies [125,131,133,134] have clearly demonstrated that the valence of the buffer (I, II or III) can fundamentally change the shape of overloaded band profiles recorded at high solute concentrations. As shown in Fig. 28, any buffer or supporting salts with a valence I (e.g., buffer citrate I, pH 3.14 or supporting salts NaCl, KCl and $CaCl_2$) leads to triangle-shaped band profiles (i.e., a langmuirian profile) characteristic of convex upward isotherms for the adsorption of a positively charged compound (propranololium). When a two-times charged species is present in the solution (citrate II, pH 4.77 or $NaSO_4$) the shape of the overloaded band profiles becomes characteristic of a S-shaped isotherm, which mirrors the occurrence of adsorbate–adsorbate interactions. With a trivalent buffer (citrate III), the shape of the overloaded band profile becomes characteristic of an anti-Langmuir isotherm, exhibiting a diffuse front and a rear shock. The higher the valence, the stronger the interactions between the adsorbate molecules.

The buffer molecules, which have more than one negative charge, can possibly bond to more than one positively charged ion. They create the conditions for the analyte to develop adsorbate–adsorbate interactions, like two neutral compounds would have in neat aqueous organic solutions (ex: butyl benzene in methanol:water). This results in a convex downward curvature for the adsorption isotherm for a certain range of concentrations.

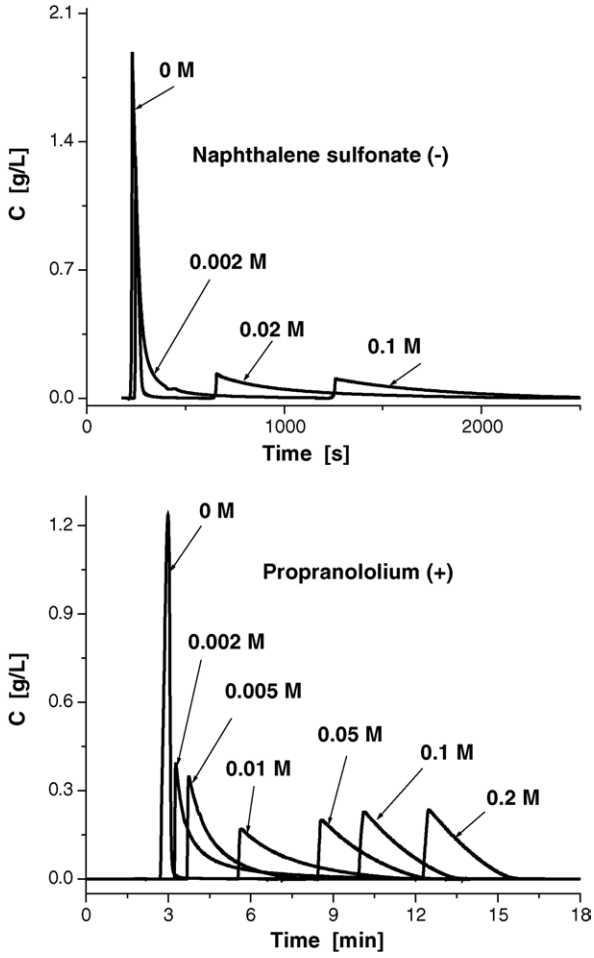


Fig. 27. Effect of the concentration of the counter-ion (NaCl) on the retention of the overloaded band profiles of naphthalene sulfonate (Na^+) and propranololium (Cl^-). Note that the retention of low concentrations are poorly affected while the retention of high concentration are drastically increased. Reproduced with permission from [132] (Fig. 4).

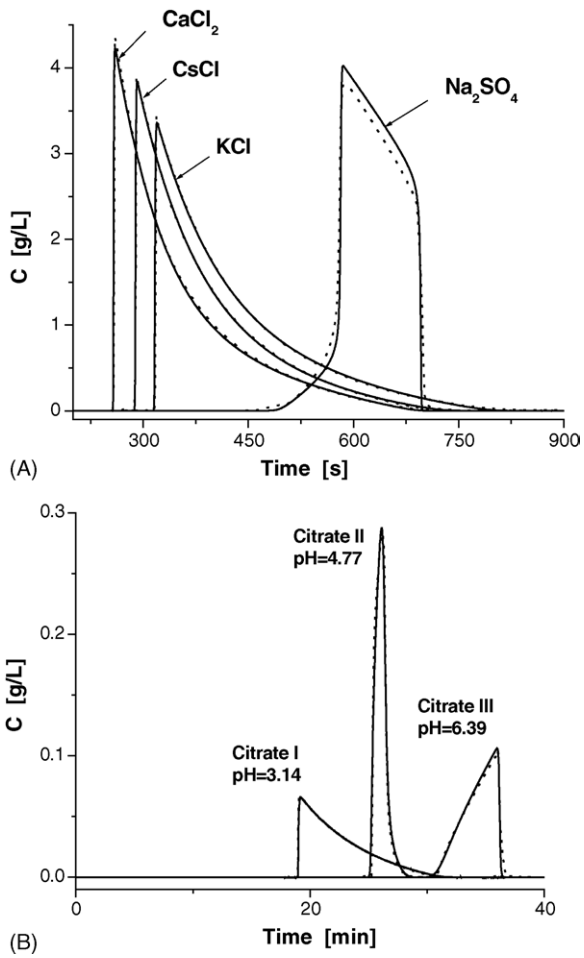


Fig. 28. Effect of the valence of the counter anion forming ion pair complexes with propranololium on the shape of the overloading profile and the nature of the isotherm. Monovalent, bivalent and trivalent anions generate langmuirian, S-shaped and antilangmuirian isotherms, respectively. (A) Supporting salts, (B) buffers. Reproduced with permission from [131] (Fig. 6) and [125] (Fig. 6) ©2004, American Chemical Society.

As a conclusion, all buffers should be expected to have different effects on isotherms, retention, and band profiles, despite the fact that they are used at the same or similar concentrations and at the same or close pH. The hydrophobicity of the co-ion, the extent of charge delocalization, and the valence of the buffer ions involved affect the retention and the adsorption isotherm of positively or negatively charged analytes, based on ion-pairing formation in the liquid phase. Because ionizable compounds also exist under the neutral ion-pair form, the saturation capacities found for ions are not drastically different from those of neutral compounds. Saturation capacities usually increase with the concentration of co-ions and the loading capacity of RPLC columns is of the same order of magnitude whether neutral or ionizable compounds are injected.

9. Conclusion

The conclusions of most works dealing with retention mechanisms in RPLC are based on the analysis of retention data acquired in linear chromatography. The interpretation of these data cannot recognize that retention depends on several independent contributions and neglects the nature and the extent of the heterogeneity of the surface of modern RPLC packing materials because linear chromatography cannot distinguish any of the individual contributions to retention. The combination of the acquisition of FA data in a wide range of concentrations, the isotherm modeling of these data, and the calculation of the AED of probe compounds demonstrates that nonlinear data are far more informative. These data are as reproducible as linear data and we have shown that the numerical parameters of the isotherm models accounting best for the data obtained with any compound are highly reproducible. Several important conclusions can be drawn from these nonlinear data.

The surface of modern RPLC adsorbents are not homogeneous but are covered with different types of adsorption sites. The low-energy type of sites are the most abundant. They are located at the interface between the mobile phase and the layer of bonded C_{18}-chains. Other types of sites seem located inside the C_{18}-bonded layer, more or less deep, which explains a somewhat stronger retention. Depending on the difference between their adsorption energy and that of the low-energy sites (between +5 and +20 kJ/moL), these quasi-partitioning sites involve interactions with the alkyl chains that are similar to the interactions taking place in solution, but which do so in the electric field of the silica adsorbent, or interactions with the siloxane or silanol groups of the silica support. The abundance of these high-energy sites depends on the size of the analyte selected. The larger the size of this analyte, the most likely the chances to reveal new high-energy adsorption sites, in which the analyte fits snugly and is well embedded. C_{18}-bonded phases could be seen as a sort of imprinted phases, which retains preferentially certain analytes that have specific geometry to interact with the C_{18} chains macro-environment. This model of RPLC silica packing materials are supported by the observations made with phenol, caffeine, and a few other molecular probes on a large number of C_{18}-bonded stationary phases. It explains well why the retention of caffeine is less than that of phenol while it has a larger molecular mass and a larger hydrophobicity. The paradox of their retention order is explained when the heterogeneity of the C_{18}-bonded layer and the different properties of the adsorption sites found are taken into account.

The retention mechanism in RPLC is based on the simultaneous adsorption and partition of the analyte on and inside the hydrophobic layer. This conclusion has already been suggested [17] but it could not be demonstrated for the lack of high concentrations data. Because FA data can be measured in the linear range and up to concentrations close to the solubility of the analytes, they permit the identification of the several contributions to the retention of analytes. Their use demonstrates clearly the validity of these early suggestions. In addition, FA permits a direct quantitation of the relative abundance, of the adsorption energy, and of the adsorption constants of each type of sites, and of the range of concentrations within which each of these types fills up. The presence of high-

energy sites that are filled at very low concentrations is a serious source of troubles because they strongly affect retention times at low concentrations and prevent from acquiring reproducible retention data for an analyte since, even at very low concentrations, its retention time may depend strongly on the sample concentration [72].

The adsorption mechanism on RPLC packing materials depends strongly on the polarity of the analyte. In contrast with conventional wisdom, most adsorption isotherms in RPLC are not accounted for by a Langmuir model. Many are not even convex upward isotherms as they seem to be in normal phase HPLC. The data bank presented in this review suggests, on a statistical basis that polar compounds tend to have convex upward isotherms while convex downward isotherms were systematically observed for apolar or low-polarity compounds. Adsorbate–adsorbate interactions are the main reason for this anti-langmuirian behavior. A large variety of adsorption isotherms were observed that have a behavior intermediate between these two extremes (i.e., S-shaped isotherms), depending on the mobile phase concentration at which adsorbate–adsorbate interactions become significant.

Although the same model best accounts for data obtained with a given compound on a wide variety of stationary phases, under many different sets of experimental conditions,[2] the numerical values of the coefficients of these adsorption isotherm are very sensitive to these conditions. Saturation capacities, equilibrium constants, and interaction coefficients depend much on the temperature, the pressure, and the mobile phase composition. From a preparative point of view, the knowledge of the evolution of the isotherm parameters with the experimental conditions is critical to maximize the production rate. Particularly important from this point of view is the large increase of the column loadability that is observed when acetonitrile is used instead of methanol as the organic modifier. This observation is likely to be related to the adsorption behavior of acetonitrile on C_{18}-bonded stationary phases. A thick adsorbed multilayer phase of acetonitrile forms between the bonded alkyl chains and the mobile phase and permits the adsorption of larger amounts of feed than with methanol, which simply forms an adsorbed monolayer on the top of the C_{18} chains.

Finally, this work brings a new general framework of consistent interpretation of the observations made when the experimental condition of the chromatographic process are changed in RPLC. It sheds new light on the true nature of the adsorbent surface of modern packing materials. It delineates the thrust of the remaining efforts that should be made to improve the quality of these materials, particularly in terms of the control of their heterogeneity and selectivity. Complementing other modes of characterization of packing materials for chromatography (NMR, FTIR, fluorescence), FA measurements provide a consistent, detailed description of the behavior of C_{18}-bonded stationary phases. It provides a quantitative description of the surface heterogeneity, hence, a reliable tool of investigations of the influence of all the parameters of the manufacturing process of RPLC packing materials on their performance.

[2] If we realize that a bi-Langmuir isotherm is but a bi-Moreau isotherm with $I_i = 0$.

Acknowledgments

This work was supported in part by grant CHE-02-44693 of the National Science Foundation, by Grant DE-FG05-88-ER-13869 of the US Department of Energy, and by the cooperative agreement between the University of Tennessee and the Oak Ridge National Laboratory. We thank H. Liliedahl and L. Torstensson (Eka Nobel, Bohus, Sweden) for the generous gift of the Kromasil-C_{18} columns, U.D. Neue and M. Kele for the gift of the Symmetry-C_{18}, XTerra-C_{18}, and Resolve-C_{18} columns and T. Farkas for the gift of the Luna-C_{18} column used in this work.

References

[1] K.K. Unger, Porous Silica, Elsevier, Amsterdam, New York, 1979.
[2] U.D. Neue, HPLC Columns. Theory, Technology, and Practice, Wiley-VCH, New York, 1997.
[3] S.D. Rogers, J.G. Dorsey, J. Chromatogr. A 892 (2000) 57.
[4] U.D. Neue, in: R.A. Meyers (Ed.), Encyclopedia of Analytical Chemistry, John Wiley & Sons, Chichester, 2000.
[5] W.R. Melander, Cs. Horváth, Cs. Horváth (Eds.), High Performance Liquid Chromatography, Academic, New York, 1980, p. 1132.
[6] Cs. Horváth, W.R. Melander, I. Molnar, J. Chromatogr. 125 (1976) 129.
[7] L.C. Sander, S.A. Wise, Anal. Chem. 56 (1984) 504.
[8] S.A. Wise, L.C. Sander, J. High Resolut. Chromatogr. Chromatogr. Commun. 8 (1985) 248.
[9] K.B. Sentell, J.G. Dorsey, J. Chromatogr. 461 (1989) 193.
[10] H. Colin, G. Guiochon, J. Chromatogr. 158 (1978) 183.
[11] C.H. Lochmuller, H.H. Hangac, D.R. Wilder, J. Chromatogr. Sci. 19 (1981) 130.
[12] C.H. Lochmuller, M.L. Hunnicutt, J.F. Mullaney, J. Phys. Chem. 89 (1985) 5770.
[13] P.C. Sadek, P.W. Carr, J. Chromatogr. 288 (1984) 25.
[14] G.E. Berendsen, K.A. Pikaart, L. deGalan, J. Chromatogr. 52 (1980) 1990.
[15] A. Tchapla, H. Colin, G. Guiochon, Anal. Chem. 56 (1984) 621.
[16] A. Tchapla, S. Heron, H. Colin, G. Guiochon, Anal. Chem. 60 (1988) 1443.
[17] J.G. Dorsey, K.A. Dill, Chem. Rev. 89 (1989) 331.
[18] K.A. Dill, J. Phys. Chem. 91 (1987) 1980.
[19] M.J. Wirth, H.O. Fatunmbi, Anal. Chem. 64 (1992) 2783.
[20] L.C. Sander, J.B. Callis, L.R. Field, Anal. Chem. 55 (1983) 1068.
[21] M. Raitza, M. Herold, A. Ellwanger, G. Gauglittz, K. Albert, Macromol. Chem. Phys. 201 (2000) 825.
[22] M. Raitza, J. Wegman, S. Bachman, K. Albert, Angew. Chem. Int. Edu. 39 (19) (2000) 3486.
[23] M. Pursch, L.C. Sander, H.-J. Egelhaaf, M. Raitza, S.A. Wise, D. Oelkrug, K. Albert, J. Am. Chem. Soc. 121 (1999) 3201.
[24] L.R. Snyder, J.W. Dolan, P.W. Carr, J. Chromatogr. A 1060 (2004) 77.
[25] A. Wang, P.W. Carr, J. Chromatogr. A 965 (2002) 3.
[26] N.S. Wilson, M.D. Nelson, J.W. Dolan, L.R. Snyder, R.G. Wolcott, P.W. Carr, J. Chromatogr. A 961 (2002) 171.
[27] P.W. Carr, Microchem. J. 48 (1993) 4.
[28] G. Götmar, B.J. Stanley, T. Fornstedt, G. Guiochon, Langmuir 18 (1984) 611.
[29] G. Götmar, T. Fornstedt, G. Guiochon, Chirality 12 (2000) 558.
[30] G. Götmar, T. Fornstedt, G. Guiochon, Anal. Chem. 72 (2000) 3908.

[31] T. Fornstedt, G. Götmar, M. Andersson, G. Guiochon, J. Am. Chem. Soc. 121 (1999) 1164.
[32] T. Fornstedt, G. Götmar, M. Andersson, G. Guiochon, Chirality 10 (1998) 375.
[33] T. Fornstedt, P. Sajonz, G. Guiochon, J. Am. Chem. Soc. 119 (1997) 1254.
[34] K. Mihlbachler, K. Kaczmarski, A. Seidel-Morgenstern, G. Guiochon, J. Chromatogr. A 955 (2002) 35.
[35] F. Gritti, G. Guiochon, J. Colloid Interface Sci. 264 (2003) 43.
[36] H. Kim, G. Guiochon, Anal. Chem. 77 (2005) 93.
[37] G. Guiochon, S.G. Shirazi, A.M. Katti, Fundamentals of Preparative and Nonlinear Chromatography, Academic Press, Boston, MA, 1994.
[38] D.H. James, C.S.G. Phillips, J. Chem. Soc. (1954) 1066.
[39] G. Schay, G. Szekely, Acta Chim. Hung. 5 (1954) 167.
[40] A. Seidel-Morgenstern, J. Chromatogr. A 1037 (2004) 255.
[41] F. Gritti, G. Guiochon, J. Chromatogr. A 1070 (2005) 1.
[42] F. Gritti, G. Guiochon, J. Chromatogr. A 1070 (2005) 13.
[43] F. Gritti, G. Guiochon, J. Chromatogr. A 1075 (2005) 117.
[44] F. Gritti, G. Guiochon, J. Chromatogr. A, in press (JCA-05-876).
[45] F. Gritti, G. Guiochon, J. Chromatogr. A 1028 (2004) 75.
[46] M. Jaroniec, R. Madey, Physical Adsorption on Heterogeneous Solids, Elsevier, Amsterdam, The Netherlands, 1988.
[47] D.S. Jovanovic, Colloid Polym. Sci. 235 (1969) 1203.
[48] M. Moreau, P. Valentin, C. Vidal-Madjar, B.C. Lin, G. Guiochon, J. Colloid Interface Sci. 141 (1991) 127.
[49] S. Brunauer, P.H. Emmet, E. Teller, J. Am. Chem. Soc. 60 (1938) 309.
[50] R.J. Umpleby II, S.C. Baxter, Y. Chen, R.N. Shah, K.D. Shimizu, Anal. Chem. 73 (2001) 4584.
[51] J. Tóth, Adsorption, M. Dekker, New York, NY, 2002.
[52] B.J. Stanley, S.E. Bialkowski, D.B. Marshall, Anal. Chem. 659 (1994) 27.
[53] D.M. Ruthven, Principles of Adsorption and Adsorption Processes, Wiley, New York, NY, 1984.
[54] M. Suzuki, Adsorption Engineering, Elsevier, Amsterdam, The Netherlands, 1990.
[55] P.W. Danckwerts, Chem. Eng. Sci. 2 (1953) 1.
[56] P. Rouchon, P. Valentin, M. Schonauer, C. Vidal-Madjar, G. Guiochon, J. Phys. Chem. 88 (1985) 2709.
[57] P. Rouchon, M. Schonauer, P. Valentin, G. Guiochon, Sep. Sci. Technol. 22 (1987) 1793.
[58] G. Guiochon, S. Golshan-Shirazi, A. Jaulmes, Anal. Chem. 60 (1988) 1856.
[59] D.W. Marquardt, J. Soc. Appl. Math. 11 (1963) 431.
[60] S. Brunauer, L.S. Deming, W.E. Deming, E. Teller, J. Am. Chem. Soc. 62 (1940) 1723.
[61] M.H. Abraham, J. Andonian-Haftvan, G.S. Whiting, A. Leo, R.S. Taft, J. Chem. Soc., Perkin Trans. 2 (1994) 1777.
[62] A. Cavazzini, G. Bardin, K. Kaczmarski, P. Szabelski, M. Al-Bokari, G. Guiochon, J. Chromatogr. A 957 (2002) 111.
[63] F. Gritti, W. Piatkowski, G. Guiochon, J. Chromatogr. A 978 (2002) 81.
[64] F. Gritti, G. Guiochon, J. Chromatogr. A 1008 (2003) 13.
[65] F. Gritti, G. Guiochon, J. Chromatogr. A 1008 (2003) 23.
[66] F. Gritti, G. Guiochon, J. Chromatogr. A 1028 (2004) 121.
[67] S. Golshan-Shirazi, G. Guiochon, Anal. Chem. 60 (1988) 2634.
[68] I. Langmuir, J. Am. Chem. Soc. 40 (1918) 1361.
[69] F. Gritti, G. Guiochon, Anal. Chem. 75 (2003) 5726.
[70] F. Gritti, G. Guiochon, J. Chromatogr. A, in press (JCA-C996).
[71] F. Gritti, G. Guiochon, J. Chromatogr. A 1090 (2005) 39.
[72] F. Gritti, G. Guiochon, Anal. Chem. 77 (2005) 1020.
[73] M. Kele, G. Guiochon, J. Chromatogr. A 855 (1999) 423.
[74] F. Gritti, G. Guiochon, J. Chromatogr. A 1003 (2003) 43.
[75] A. Felinger, F. Gritti, G. Guiochon, J. Chromatogr. A 1024 (2004) 21.
[76] T. Ikegami, E. Dicks, H. Kobayashi, H. Morisaka, D. Tokuda, K. Cabrera, K. Hosoya, N. Tanaka, J. Sep. Sci. 27 (2004) 1292.
[77] F. Gritti, G. Guiochon, J. Chromatogr. A 1021 (2003) 25.
[78] F. Gritti, G. Guiochon, J. Chromatogr. A 1028 (2004) 105.
[79] F. Gritti, G. Götmar, B.J. Stanley, G. Guiochon, J. Chromatogr. A 988 (2003) 185.
[80] J. Tóth, Acta Chem. Hung. 32 (1962) 31.
[81] J. Tóth, Acta Chem. Hung. 69 (1971) 311.
[82] D. Graham, J. Phys. Chem. 57 (1953) 665.
[83] A.B. Scholten, J.W. de Haan, H.A. Claessens, L.J.M. Van de Ven, C.A. Cramers, Langmuir 12 (1996) 4741.
[84] A. Méndez, E. Bosch, M. Rosés, U.D. Neue, J. Chromatogr. A 986 (2003) 33.
[85] F. Gritti, G. Guiochon, J. Chromatogr. A 995 (2003) 37.
[86] F. Gritti, G. Guiochon, J. Chromatogr. A 1010 (2003) 153.
[87] F. Gritti, A. Felinger, G. Guiochon, J. Chromatogr. A 1017 (2003) 45.
[88] K. Miyabe, G. Guiochon, J. Phys. Chem. B 106 (2002) 8898.
[89] K. Kimata, K. Iwaguchi, S. Onishi, K. Jinno, R. Eksteen, K. Hosaya, M. Araki, N. Tanaka, J. Chromatogr. Sci. 27 (1989) 721.
[90] H. Engelhart, M. Jungheim, Chromatographia 29 (1990) 59.
[91] K. Schmidt-Rohr, H.W. Spiess, Multidimensional Solid-State NMR and Polymers, Academic Press, London, 1994.
[92] R. Brindle, M. Pursch, K. Albert, Solid State Nucl. Magn. Reson. A 6 (1996) 251.
[93] M. Raitza, M. Herold, A. Ellwanger, G. Gauglittz, K. Albert, Macromol. Chem. Phys. 201 (2000) 825.
[94] M. Raitza, J. Wegman, S. Bachman, K. Albert, Angew. Chem. Int. Edu. 39 (2000) 3486.
[95] M. Pursch, L.C. Sander, H.-J. Egelhaaf, M. Raitza, S.A. Wise, D. Oelkrug, K. Albert, J. Am. Chem. Soc. 121 (1999) 3201.
[96] F. Gritti, G. Guiochon, J. Chromatogr. A 1069 (2005) 31.
[97] V.L. McGuffin, C.E. Evans, J. Microcol. Sep. 3 (1991) 513.
[98] G. Guiochon, M.J. Sepaniak, J. Chromatogr. 606 (1992) 148.
[99] C.E. Evans, J.A. Davis, Anal. Chim. Acta 397 (1999) 163.
[100] D. Zhou, X. Liu, K. Kaczmarski, A. Felinger, G. Guiochon, Biotechnol. Prog. 19 (2003) 945.
[101] P. Szabelski, A. Cavazzini, K. Kaczmarski, X. Liu, J. Van Horn, G. Guiochon, J. Chromatogr. A 950 (2002) 41.
[102] X. Liu, D. Zhou, P. Szabelski, G. Guiochon, Anal. Chem. 75 (2003) 3999.
[103] X. Liu, D. Zhou, P. Szabelski, G. Guiochon, J. Chromatogr. A 988 (2003) 205.
[104] H. Kim, F. Gritti, G. Guiochon, J. Chromatogr. A 1049 (2004) 25.
[105] F. Gritti, G. Guiochon, J. Chromatogr. A 1043 (2004) 159.
[106] A. Seidel-Morgenstern, G. Guiochon, Chem. Eng. Sci. 48 (1993) 2787.
[107] L. Gueorguieva, D. Beltscheva, D. Antos, A. Seidel-Morgenstern, G. Guiochon, Fundamentals of Adsorption, Japanese Adsorption Society, 2002.
[108] L.A. Cole, J.G. Dorsey, Anal. Chem. 62 (1990) 16.
[109] A.P. Schellinger, D.R. Stoll, P.W. Carr, Anal. Chem. 1064 (2005) 143.
[110] M. Rosés, F. Rived, E. Bosch, J. Chromatogr. A 867 (2000) 45.
[111] Y.V. Kazakevich, R. Lobrutto, F. Chan, T. Patel, J. Chromatogr. A 913 (2001) 75.
[112] Y.V. Kazakevich, R. Lobrutto, R. Wivilecchia, J. Chromatogr. A 1064 (2005) 9.
[113] F. Gritti, G. Guiochon, Anal. Chem. 77 (2005) 4257.
[114] F.F. Cantwell, S. Puon, Anal. Chem. 51 (1979) 623.
[115] I. Hägglund, J. Ståhlberg, Anal. Chem. 60 (1988) 1958.
[116] I. Hägglund, J. Ståhlberg, J. Chromatogr. A 761 (1997) 3.
[117] I. Hägglund, J. Ståhlberg, J. Chromatogr. A 761 (1997) 13.
[118] Cs. Horváth, W.R. Melander, I. Molnar, P. Molnar, Anal. Chem. 49 (1977) 2295.
[119] A. Tilly-Melin, Y. Askemark, K.-G. Wahlund, G. Schill, Anal. Chem. 51 (1979) 976.
[120] A. Sokolowski, Chromatographia 22 (1986) 168.
[121] A. Sokolowski, Chromatographia 22 (1986) 177.
[122] F. Gritti, G. Guiochon, J. Chromatogr. A 1028 (2004) 197.
[123] G. Götmar, T. Fornstedt, M. Andersson, G. Guiochon, J. Chromatogr. A 905 (2001) 3.
[124] D.M. McCalley, Anal. Chem. 75 (2003) 3404.
[125] F. Gritti, G. Guiochon, Anal. Chem. 76 (2004) 7310.
[126] U.D. Neue, T. Wheat, J. Mazzeo, C. Mazza, J. Cavanaugh, F. Xia, D. Diehl, J. Chromatogr. A 1030 (2004) 123.
[127] U.D. Neue, C. Phoebe, K. Tran, Y.-F. Cheng, Z. Lu, J. Chromatogr. A 925 (2001) 49.
[128] F. Gritti, G. Guiochon, J. Chromatogr. A 1033 (2004) 43.
[129] F. Gritti, G. Guiochon, J. Chromatogr. A 1033 (2004) 57.

[130] F. Gritti, G. Guiochon, J. Chromatogr. A 1047 (2004) 33.
[131] F. Gritti, G. Guiochon, Anal. Chem. 76 (2004) 4779.
[132] F. Gritti, G. Guiochon, J. Chromatogr. A 1048 (2004) 1.
[133] F. Gritti, G. Guiochon, J. Chromatogr. A 1038 (2004) 53.
[134] F. Gritti, G. Guiochon, J. Chromatogr. A 1041 (2004) 63.
[135] F. Gritti, G. Guiochon, J. Chromatogr. A 1098 (2005) 82.
[136] F. Gritti, W. Piatkowski, G. Guiochon, J. Chromatogr. A 983 (2003) 51.
[137] F. Gritti, G. Guiochon, J. Chromatogr. A, in press (JCA-05-633).
[138] F. Gritti, G. Guiochon, J. Chromatogr. A, in press (JCA-05-895).